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INTRODUCTION

The *PTEN/MMAC/TEP-1* tumor suppressor gene (hereafter referred to as *PTEN*) is a target of somatic mutation in prostate cancer as well as in endometrial cancer, glioblastoma and melanoma (reviewed in (Sansal and Sellers, 2004)). Biallelic loss of *PTEN* has been demonstrated in both primary and metastatic prostate tumors (reviewed in (Sansal and Sellers, 2004)). In metastatic disease, *PTEN* loss approaches 50%-60% (Suzuki et al., 1998). Together, these data suggest that loss of PTEN is an important step for those prostate tumors associated with a lethal outcome. Moreover, the loss of PTEN has been intimately linked to deregulation of the PI3K pathway connecting growth and survival signals both to the regulation of the mTOR kinase as well as to the regulation of the FOXO transcription factors. A significant effort is now being expended in the pharmaceutical industry in trying to develop regulators of the PI3K pathway (or more specifically inhibitors) that can reverse the molecular consequences of PTEN loss.

Our group was among the first to publish data that FOXO proteins are aberrantly localized to the cytoplasm upon loss of functional PTEN and that FKHR localization is restored upon reconstitution of PTEN to PTEN null cells (Nakamura, et. al. 2000). Further, we showed that reconstitution of FOXO activity to the nucleus was sufficient to replace the tumor suppressor function of PTEN (Nakamura, et. al. 2000). Surprisingly this activity was not linked to the transactivation activity of FOXO but was linked to the ability of FOXO to repress cyclin D1 (Ramaswamy et. al. 2002). In total these data suggest that to a first approximation, small molecules that recapitulate this activity of PTEN, i.e. lead to re-distribution of FKHR from the cytoplasm to the nucleus, should lead to inhibition of cell-cycle progression and suppression of tumorigenicity of PTEN null cells.

These data led us to ask whether a novel cell-based small-molecule screen could be developed using FKHR localization as an end-point. Preliminary data showed that this was feasible and led to the discovery of novel small molecule inhibitors of the PI3K pathway (Kau et. al. 2003). Based on these results we proposed 3 specific aims:

1) To determine the mechanism of action of inhibitors that specifically re-localize FKHR to the nucleus
2) To determine the in vitro biological activity of small molecule inhibitors discovered in the FKHR screen.
3) To determine the in vivo anti-tumor efficacy of lead compounds in animal models.

Body

(please note: that since the following data are unpublished they are taken from the annual reports of 2005 and 2006)

Our discovery of small molecule inhibitors of FOXO cytoplasmic localization focused on the phenothiazine class of compounds as three distinct phenothiazines scored in the initial screen (Kau et. al. 2003 – see appendix). Initially it was thought that this might identify dopamine receptors as the relevant molecular target in this assay. Additional experiments, however, showed that unrelated dopamine receptor antagonists (such as haloperidol) failed to score in the FOXO localization assays. Phenothiazines are also known to be inhibitors of calmodulin and indeed, structurally unrelated calmodulin inhibitors or calcium chelating agents were able to recapitulate the ability of the phenothiazines to re-localize FOXO factors to the nucleus. From these data we concluded that phenothiazines, through their calmodulin antagonistic activity, can regulate the localization of FOXO.

Thioridazine decreases phospho-Ser473 and phospho-Thr308 Akt levels.

In order to further clarify the phosphoinositide-3 kinase pathway inhibitory activity of phenothiazines, the phosphorylation state of Akt and its downstream proteins were examined after treatment with thioridazine or other phenothiazine derivatives in both PTEN wild-type and PTEN mutant cell lines. To this end, extracts were prepared from PTEN null LNCaP cells grown in complete media at different time points after treatment with 20 μM thioridazine (Fig. 1). Treatment with thioridazine decreased phospho-Thr308-Akt levels from 1 hr to 8 hrs after treatment with peak activity seen at the 5-hour time point thioridazine. Phosphorylation of Ser473-Akt levels were abolished with a similar time course. To determine whether the loss of Akt phosphorylation was associated with loss of phosphorylation of downstream pathway effectors, protein extracts were probed with antisera recognizing phosphorylated GSK3α/β and phosphorylated S6 ribosomal protein. Phosphorylation of GSK3α/β and S6 ribosomal protein were diminished with a similar time course.

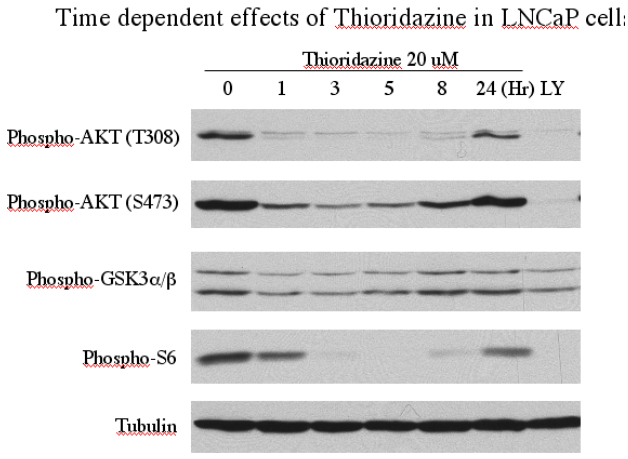

Figure 1: Time course of PI3K pathway inhibition after treatment with thioridazine (see text).

Next, to determine whether such inhibition was dose dependent, LNCaP cells were treated with increasing concentration of thioridazine for 5 hrs and the protein extracts again were immunoblotted with the relevant phosphospecific antisera (Fig. 2). Treatment with thioridazine decreased the levels of phospho-Thr308-Akt, phospho- Ser473-Akt, phosphorylated GSK3 and phosphorylated S6 ribosomal in a dose dependent manner. To determine the cell based concentration of thioridizaine leading to 50% diminishment of Akt activity (Cellular IC50) the phosphorylation of Akt was quantified by determining the optical density of bands of phospho-Ser473- and phospho-Thr308-Akt on immunoblot (data not shown) analyzed by NIH-Image software and normalized with anti-GSK3α. The thioridazine concentration producing 50% inhibition (IC50) of phospho-Ser473- and phospho-Thr308-Akt levels were 9.6 and 11.7 μM (data not shown) respectively.

Phenothiazines can act up and downstream of PTEN.

A prediction of these data is that PTEN null cells might be preferentially sensitized to cellular growth inhibitory effects of phenothiazine. To try and determine whether this was the case, we next examined the effect of phenothiazines in two pairs of cell lines, LNCaP and DU145 prostate cancer cell lines and 786-O and ACHN renal carcinoma cell lines (data not shown). LNCaP and 786-O cells fail to express any full length of PTEN protein, but DU145 and ACHN cells retain wild-type PTEN alleles and express an intact PTEN protein (Ramaswamy et al., 1999). In PTEN null cells, as previously noted by numerous groups including our own, Akt activity and phosphorylation of substrates such as FOXOs (FOXO3A) and GSK3 are substantially lower when compared to PTEN null cells. Treatment with 20 μM thioridazine for 5 hours decreased the phosphorylation of Akt, FKHRL1, and GSK3in both PTEN-null cells (LNCaP and 786-O).

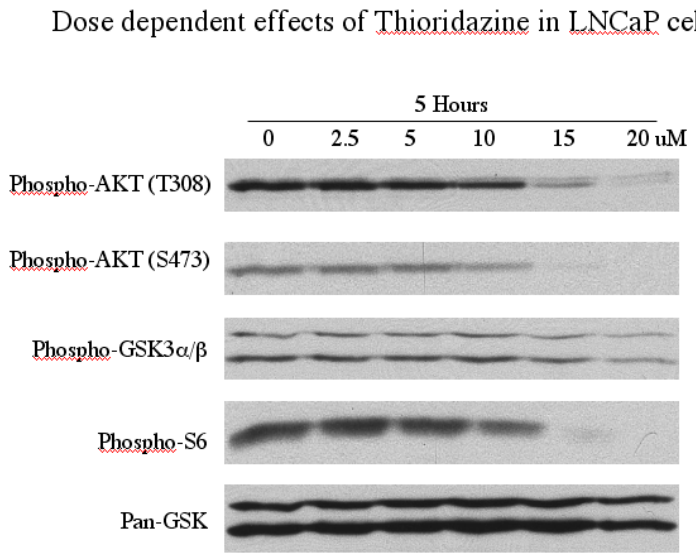

Figure 2: Dose-dependent inhibition of PI3K pathway signaling by phenothiazines (see text).

The phosphorylation state of p70 S6 kinase (p70 S6K) and S6 ribosomal protein were also inhibited. Here a notable finding was that this inhibition by thioridazines was NOT directly correlated with PTEN status. Specifically, under plating conditions where cells were plated at low-density PTEN wild-type DU145 and ACHN cells showed hyperphosphorylation of S6 ribosomal protein and p70S6K, which could be rapidly blocked by treatment with Thioridazine (data not shown). Thus, thioridazine treatment led to decreased phosphorylation of p70 S6K and S6 ribosomal protein in all PTEN-null and PTEN wild-type cell lines tested, LNCaP, 786-O, DU145 and ACHN cells.

The lack of robust Akt activation in the PTEN wild-type cells, and the inhibitory effect of thioridazine seen in the absence of Akt activity, suggests that phenothiazines may block pathway activation both upstream and downstream of Akt. Together with previous data linking the activity of phenothiazimes to calmodulin inhibition, these data raise the possibility that phenothiazines interdict a calmodulin dependent regulatory mechanism required both upstream and downstream of Akt in the PI3K pathway. Here, the regulation of PDK1 stands out as an attractive hypothesis for this novel dual mechanism of pathway inhibition.

There is significant emerging data supporting a positive feedback loop to AKT activation which can be induced by mTOR inhibition. Since this feedback loop might antagonize the effects of mTOR inhibition, a bispecific inhibitor PI3K pathway might be attractive. IC50 for growth showed that PTEN cells remain

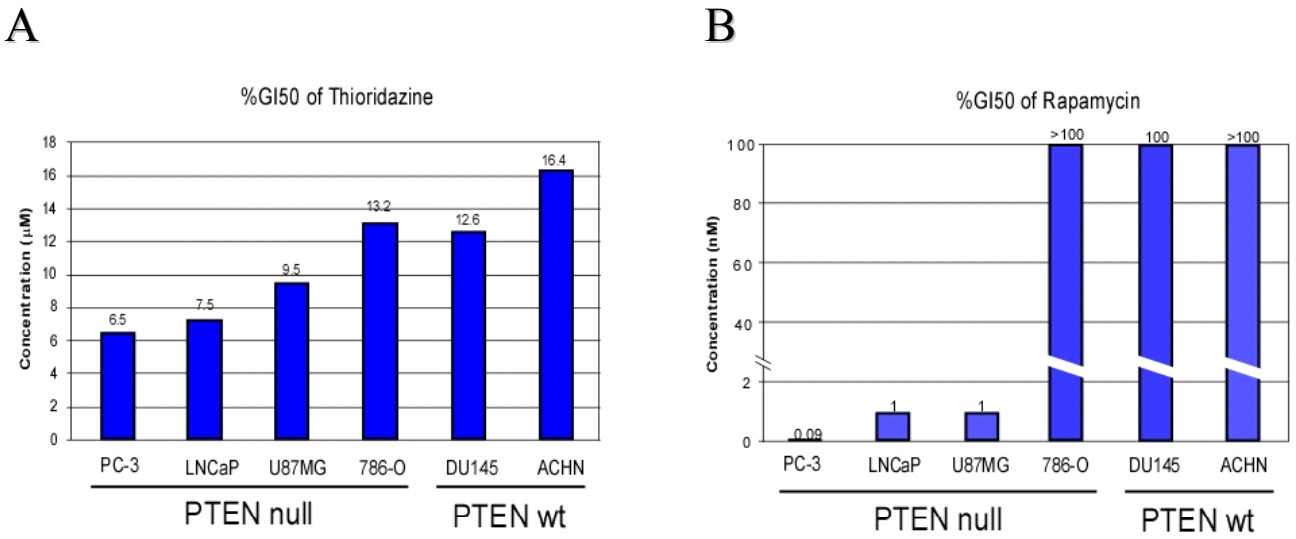

Figure 3: The indicated cell lines were treated with increasing concentrations of either thioridazine (A) rapamycn (B) and the concentration resulting in a 50% reduction in the rate of cell growth was determined and is shown on the Y-axis. In parallel control cells were treated with vehicle (DMSO).

sensitized to the growth inhibitory effects of phenothiazines compared to normal cells. PTEN null cells are known to be preferentially sensitized to mTOR inhibition (Aoki et al., 2001; Neshat et al., 2001; Podsypanina et al., 2001). To determine whether PTEN null cells similarly might be sensitized to growth inhibitory effects of phenothiazines, we tested the growth inhibitory effects of several phenothiazines, thioridazine, trifluoperazine, chlorpromazine, fluphenazine, and prochlorperazine, in the PTEN null PC-3 and U87MG cells. Among the tested phenothiazines, thioridazine showed most potent growth inhibitory effect in both cell lines (data not shown). To examine the inhibitory effect of thioridazine on the growth of PTEN-wild type vs. PTEN-null cells, we treated 6 cell lines that have been defined the PTEN status with varying dose of thioridazine. The concentration of thioridazine required for 50% growth inhibition (%GI50) for PTEN-null cell lines were lesser than for PTEN-wt cell lines except 786-O cells (Figure 3 and data not shown). We tested the growth inhibitory effects of rapamycin in these cell lines and compared the growth inhibitory effects of thioridazine and rapamycin. Rapamycin is known as a potent mTOR inhibitor, a downstream protein kinase in the Akt pathway. Rapamycin showed strong growth inhibitory effect in PTEN-null cells but 786-O cells showed resistance to rapamycin (data not shown). Rapamycin sensitive cell lines, PC-3, LNCaP and U87MG cells, were relatively more sensitive to thioridazine than rapamycin resistant cell lines. 786-O which showed rapamycin resistance in spite of PTEN-null status was also relatively resistant to thioridazine. This line in the NCI60 is reported as both sensitive, in one set of experiments, and insensitive in another. These similar sensitivity patterns of various cell lines which have different PTEN state to rapamycin and thioridazine would suggest that the growth inhibitory effect of thioridazine is likely linked to its PI3K pathway inhibitory activity. If thioridazines have added therapeutic benefits resulting from the upstream inhibition of Akt, then the ability to block Akt phosphorylation might be expected to enhance the anti-cancer activity of rapamycin. On the other hand if most of the growth inhibitor effects result from inhibition of mTOR signaling we

would expect non-synergistic or even non-additive activity. We tested the combination effect of thioridazine and rapamycin in LNCaP and DU145 cells (data not shown). We treated cells with the mixture of serially diluted concentration of rapamycin and fixed concentrations of thioridazine. The combination did not induce synergistic growth inhibition or result in any remarkable differences in sensitivity to rapamycin in LNCaP and DU145 cells.

Is there synergy with rapamycin?

The data suggesting that thioridazine could act both upstream and downstream of AKT raised the possibility that thioridazine and/or phenothiazines in general might act to block a well-described feedback loop in the PI3K/mTOR pathway. This feedback loop dramatically unveiled in TSC null fibroblasts results in up regulation of AKT activity upon down regulation of mTOR activity. Thus mTOR inhibitors such as rapamycin while inhibiting S6K activity can lead to increased AKT activity. We asked whether or not thioridazine could block this and hence act synergistically with rapamycin. Representative of these results are those shown in Figure 4. Here we failed to demonstrate synergy between the phenothiazine thioridazine and rapamycin.

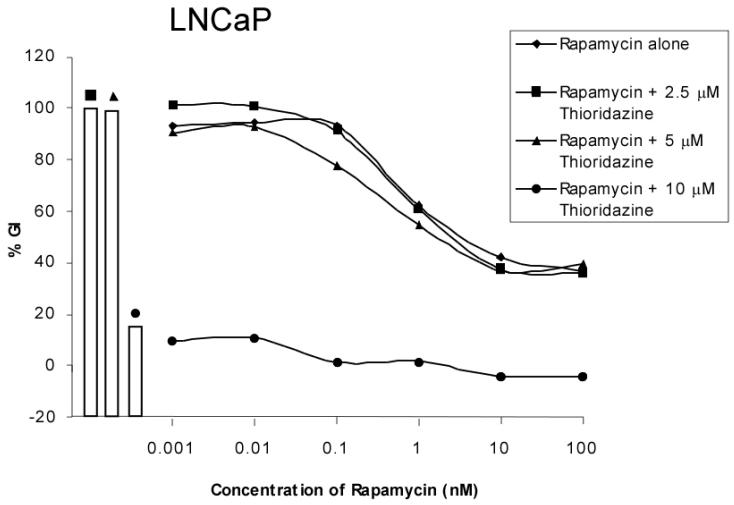

Figure 4: Thiordiazine does not synergize with rapamycin. PTEN null LNCaP cells were treated with thioridazine alone at 2.5, 5 and 10 μm (bars) or the same concentrations of thioridazine with increasing concentrations of rapamycin. No synergy was demonstrated.

In vivo testing of phenothiazines in the Tg-AKT murine PIN model.

The focus for this past year was to determine whether phenothiazines were capable of inhibiting AKT signaling and inducing an appropriate phenotypic response in in vivo models. To this end we have used two different types of models. First, we have previously published work showing that a model in which activated human AKT1 is expressed in the prostate robustly develops PIN with very high penetrance (Majumder et. al. , 2003). Moreover, we have also published that in this model treatment with the rapamycin derivative RAD001 results in complete reversion of the PIN phenotype over at 15 day time course (Majumder et. al. 2004). This model thus allows us to test phenothiazines in a model that is known to be AKT dependent. To this end cohorts of mice were treated with either placebo or thioridazine given 10mg/kg for three doses. Mice were then sacrificed and the ventral lobe of the prostate was harvested and analyzed for AKT activity by staining with anti-phospho AKT antibodies. As shown in figure 5 – we observed no change in the level of AKT phosphorylation, nor any change in S6 phosphorylation (not shown). One likely possibility is that the dose of thioridazine administered to these animals was inadequate to achieve the concentration (or exposure) required to inhibit the pathway. While we do not have access to the tools required to assess the specific plasma AUC or tissue AUC of thioridazine we did explore increasing doses of the compound. Unfortunately at doses higher than 10

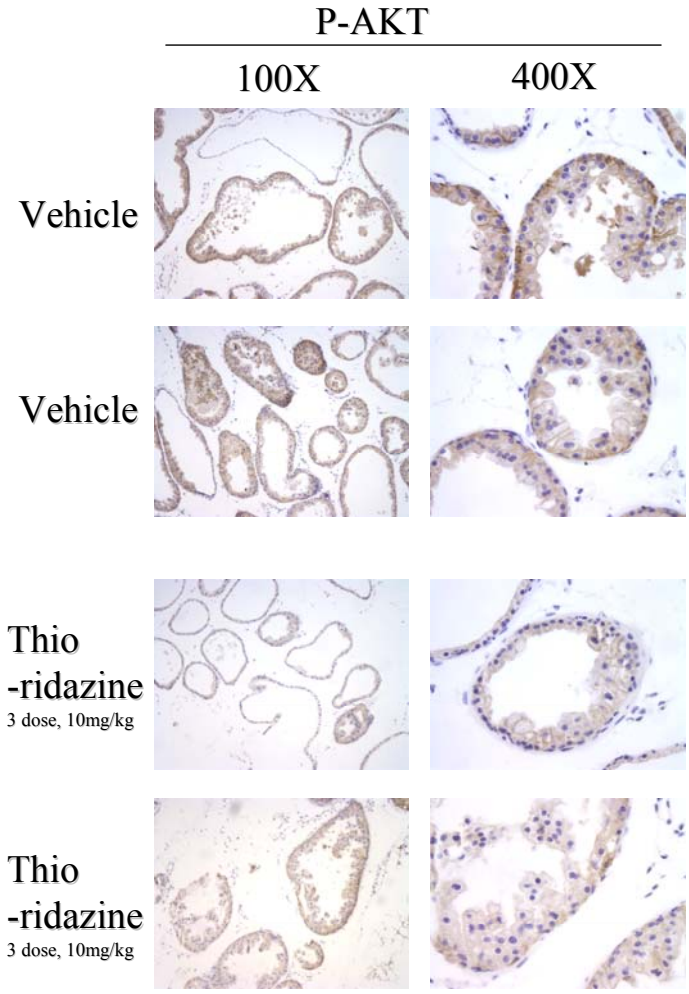

Figure 5: Thioridazine does not inhibit AKT phosphorylation in the Tg-AKT murine model. Cohorts of Tg-AKT mice were either treated with 3 doses of thoridazine or vehicle control as indicated. Sections prepared after harvesting the ventral prostate were stained with anti-Phospho-AKT antisera.

mg/kg the mice were either lethargic or the dose was toxic and lethal to the animals. Indeed, it was difficult to administer more than three doses of the compound without encountering excess toxicity in the mice.

It is formally possible that such results might be obtained if the activation of Myr-HA-AKT1 was not subjected to regulation or inhibition by phenothiazines. To determine whether this was the case, RAT1A cells were transduced with a retrovirus directing the expression of Myr-HA-AKT1. Stable polyclonal pools of cells were derived and exhibited all of the hallmarks of transformation including growth in soft-agar and the induction of tumor growth in nude mice (data not shown). These cells were then treated in vitro with either vehicle alone or increasing concentrations of thioridazine. Here we were able to demonstrate robust inhibition of phosphorylation of Myr-HA-AKT1 and the inhibition of subsequent downstream signaling consequences as indicated by inhibition of phosphorylation of ribosomal protein S6 (Figure 6). From these data we conclude that thioridazine can inhibit PI3K signaling in vitro but there is no therapeutic index that allows inhibition of PI3K in murine models.

In vivo assessment of thioridazine in LNCaP xenografts.

The data above suggest that there may be limited ability to use phenothiazines as inhibitors of PI3K pathway inhibitors in vivo likely due to the relative low potency and also the dose-limiting toxicity (somnolence) linked to inhibition of dopamine receptor signaling. We tried one additional model where LNCaP xenografts were established in nude mice and again cohorts were treated with either vehicle alone or thioridazine administered as shown in figure 5. Again, we observed no inhibition of AKT phosphorylation (data not shown).

All together while the data support a role for phenothiazines in up and downstream inhibition of the PI3K signaling pathway this inhibitory activity cannot be achieved in vivo due to the dose-limiting effects of inhibition of dopamine receptors and the resulting neurological sequelae.

The identification of a natural product inhibitor of FOXO localization

Continued work resulting from the initial FOXO screen has resulted in the identification of a natural product inhibitor of the pathway. This work is described in the accompanying publication: Schroeder et al, The psammaplysenes, specific inhibitors of FOXO1a nuclear export. J Nat Prod. 2005 Apr;68(4):574-6.

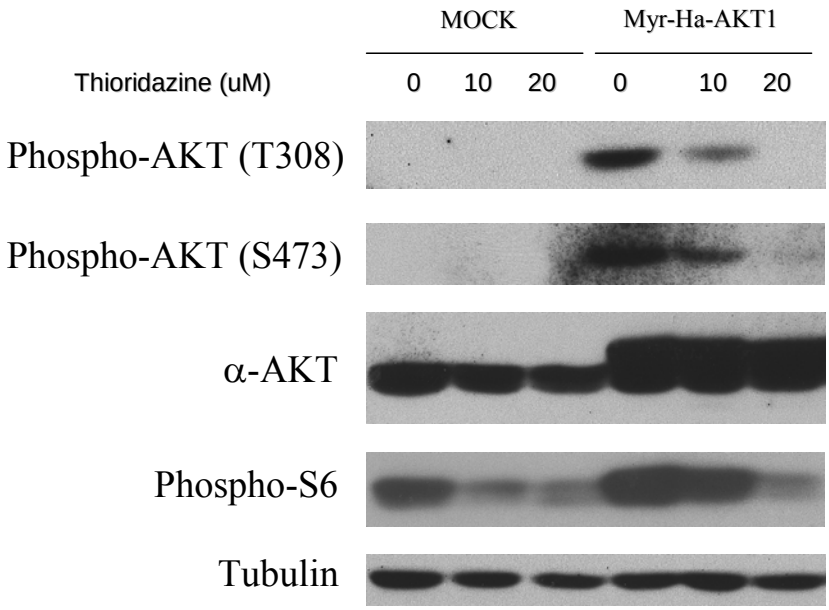

Figure 6: Thioridazine inhibits the phosphorylation of Myr-HA-AKT1 and the downstream phosphorylation of ribosomal protein S6. Myr-HA-AKT1 expressing RAT1A cells were treated with either vehicle or the indicated concentrations of Thioridazine for 6 hours. Cell lysates were prepared and separated by gel electrophoresis. The indicated protein species were detected by immunoblotting.

KEY RESEARCH ACCOMPLISHMENTS:

- Performed a screen for small molecule inhibitors of FOXO localization in PTEN null cells
- Identified Phenothiazines as inhibitors of FOXO localization through their action as calmodulin antagonists.
- In collaboration with the Clardy lab identified a novel natural product (the psammaplysenes) that inhibits FOXO cytoplasmic localization in PTEN null cells.
- Demonstrated preferential growth inhibition of PTEN null cells for the phenothiazine class of drugs.
- Showed that phenothiazines can act both upstream and downstream of Akt in the PI3K pathway.

- Showed that phenothiazines can inhibit constitutively activated AKT (activated herein using a Myristoylation signal). These data together with the dual upstream and downstream activity suggest a role in regulating PDK1 function.
- Showed that phenothiazines are unable to inhibit the AKT pathway *in vivo* in a Myr-AKT1 transgenic prostate model likely due to dose-limiting toxicity.
- Showed that phenothiazines are unable to inhibit the AKT pathway in vivo in human xenograft models again likely due to dose-limiting toxicity.

REPORTABLE OUTCOMES:

Provide a list of reportable outcomes that have resulted from this research to include:

1. Kau, T. R., Schroeder, F., Ramaswamy, S., Wojciechowski, C. L., Zhao, J. J., Roberts, T. M., Clardy, J., Sellers, W. R., and Silver, P. A.. A chemical genetic screen identifies inhibitors of regulated nuclear export of a Forkhead transcription factor in PTEN-deficient tumor cells. Cancer Cell 2003 *4*, 463-476.

2. Sansal, I., and Sellers, W. R. The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol 2004 *22*, 2954-2963.

3. Schroeder FC, Kau TR, Silver PA, Clardy J. The psammaplysenes, specific inhibitors of FOXO1a nuclear export. J Nat Prod. 2005 Apr;68(4):574-6.

4. Kau TR, Way JC, Silver PA. Nuclear transport and cancer: from mechanism to intervention. Nat Rev Cancer. 2004 Feb;4(2):106-17. Review.

5. Majumder PK and Sellers WR. Akt-regulated pathways in prostate cancer. Oncogene 2005. **24**(50): 7465-74.

Meeting Abstracts and Presentations

1. Sellers WR. 2003. ADT Innovations in Prostate Cancer Research, Cambridge, MA: invited speaker. ”Oncogenic Signaling through the PTEN/PI3K Pathway”

2. Sellers WR. 2003 Amgen Pharmaceuticals, Cambridge, MA: invited speaker ”Oncogenic Signaling through the PTEN/PI3K Pathway”

3. Sellers WR. 2003 Center for Cancer Research, National Cancer Institute, Wash., D.C.: invited speaker. “Chemical and siRNA reverse genetic Analysis of the PTEN/PI3K signaling pathway”

4. Sellers WR. 2003 Keystone Symposium on Molecular Therapeutics, Banff, Canada: Plenary speaker. “Forkhead Transcription Factors as Mediators of Tumor Suppression Downstream of PTEN”

5. Sellers WR. 2003 The First International PTEN meeting, Scottsdale, Arizona, invited speaker, "Therapeutic targeting of the PI3K/PTEN/AKT pathway.

6. Sellers WR. 2004 Protein Kinases and Cancer: The Promise of Molecular-Based Therapies, Tahoe City, California. Session Chair and invited speaker. "Oncogenic signaling through the PTEN/PI3K/Akt pathway.

7. Sellers WR. 2004 EGFR Summit, Sunny Isle Beach, FL, invited speaker. "Therapeutic targeting of the PI3K/PTEN/AKT pathway"

8. Sellers WR. 2004 AACR Annual Meeting, Orlando, FL "Therapeutic targeting of the PTEN/PI3K/Akt pathway." Invited Speaker.

CONCLUSIONS:

From these data we conclude that while phenothiazines are indeed antagonists of the PI3K pathway the doses required to achieve pathway inhibition can not be achieved in vivo likely due to dose-limiting toxicity linked to the anti-dopaminergic activity. These data argue that more potent calmodulin inhibitors would be required to progress drugs acting this mechanism into in vivo settings. From these data we do not see utility in further in vivo testing of the phenothiazines in animal models.

LIST OF PERSONNEL RECEIVING SALARY FROM THIS RESEARCH EFFORT:

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APPENDICES:

1. Kau, T. R., Schroeder, F., Ramaswamy, S., Wojciechowski, C. L., Zhao, J. J., Roberts, T. M., Clardy, J., Sellers, W. R., and Silver, P. A. (2003). A chemical genetic screen identifies inhibitors of regulated nuclear export of a Forkhead transcription factor in PTEN-deficient tumor cells. Cancer Cell 4, 463-476.

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3. Majumder PK and Sellers WR. Akt-regulated pathways in prostate cancer. Oncogene 2005. **24**(50): 7465-74.

A chemical genetic screen identifies inhibitors of regulated nuclear export of a Forkhead transcription factor in PTEN-deficient tumor cells

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Summary

The PI3K/PTEN/Akt signal transduction pathway plays a key role in many tumors. Downstream targets of this pathway include the Forkhead family of transcription factors (FOXO1a, FOXO3a, FOXO4). In PTEN null cells, FOXO1a is inactivated by PI3K-dependent phosphorylation and mislocalization to the cytoplasm, yet still undergoes nucleocytoplasmic shuttling. Since forcible localization of FOXO1a to the nucleus can reverse tumorigenicity of PTEN null cells, a high-content, chemical genetic screen for inhibitors of FOXO1a nuclear export was performed. The compounds detected in the primary screen were retested in secondary assays, and structure-function relationships were identified. Novel general export inhibitors were found that react with CRM1 as well as a number of compounds that inhibit PI3K/Akt signaling, among which are included multiple antagonists of calmodulin signaling.

Introduction

The PTEN lipid phosphatase acts as a tumor suppressor and negative regulator of PI3K/Akt-driven cell growth and survival. It antagonizes PI3K signal transduction by dephosphorylating the PI3K phosphorylation products, PI3,4,P_2 (PIP2) and PI3,4,5,P_3 (PIP3) (Maehama and Dixon, 1998). Mutations in PTEN have been implicated in Cowden Disease—a hereditary disease marked by a high predisposition for breast and thyroid cancers (Vazquez and Sellers, 2000). In addition, PTEN deficiency has been found in cancers such as glioblastoma multiforme, endometrial and prostate cancer, melanoma, and renal cell carcinoma (Kondo et al., 2001; Kong et al., 1997; Wang et al., 1997). Inhibition of the PI3K/Akt signaling pathway in PTEN null cells can control aberrant cell growth.

Mammalian members of the FOXO or Forkhead family of transcription factors include FOXO1a, FOXO3a, and FOXO4 (also known as FKHR, FKHRL1, and AFX, repectively), each of which are phosphorylation targets of Akt (Brunet et al., 1999; del Peso et al., 1999; Kops et al., 1999; Rena et al., 1999; Takaishi et al., 1999; Tang et al., 1999). These transcription factors are involved in negatively regulating cell cycle progression and cell survival (Medema et al., 2000; Nakamura et al., 2000). The phosphorylation state and subsequent subcellular localization help regulate the activity of these factors. In PTEN mutant cells, increased PIP3 levels result in constitutive activation of Akt, which phosphorylates FOXO transcription factors at multiple sites, preventing transcriptional activity and promoting nuclear export (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 1999; Rena et al., 1999). In addition to Akt, there is growing evidence that other kinases such as SGK, DYRK1A, CK1, and PAK1 are involved in FOXO phosphorylation and export (Brunet et al., 2001; Mazumdar and Kumar, 2003; Rena et al., 2002; Woods et al., 2001). Phosphorylation of the FOXO transcription factors facilitates binding with 14-3-3 proteins and export out of the nucleus (Brunet et al., 2002; Rena et al., 2001). Nuclear export of these factors is mediated by the export recep-

SIGNIFICANCE

The PTEN gene is mutated in a significant number of tumors, leading to the loss of PTEN lipid phosphatase activity and constitutive activation of PI3K/Akt signaling. It is currently unclear as to where in this pathway one might seek to intervene with a small molecule inhibitor and whether novel targets for therapy might exist. Surprisingly, an unbiased cell-based, small molecule screen based on FOXO1a localization led to the discovery of several inhibitors, including those that inhibit calmodulin. These data suggest that this approach can lead to the identification of novel lead compounds and targets for therapeutic development against tumors in which the PI3K/Akt pathway is aberrantly activated.

tor CRM1 (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 2002).

Many proteins that exit the nucleus contain short stretches of amino acids that act as nuclear export sequences (NESs) (Gerace, 1995). The NES-bearing protein is bound in the nucleus by the nuclear export receptor, CRM1, and escorted to the cytoplasm via a channel formed by the nuclear pore complex (NPC) (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). Leptomycin B (LMB) inhibits protein nuclear export mediated by CRM1 (Kudo et al., 1998). Isolated from Streptomyces, LMB covalently modifies CRM1 at a specific cysteine residue by a Michael-type addition which inhibits binding of CRM1 with the NES-containing cargo (Kudo et al., 1999). Thus, the NES-containing cargo becomes trapped in the nucleus. FOXO transcription factors are among those proteins whose nuclear export is inhibited by LMB (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 2002).

When the three known Akt phosphorylation sites on FOXO1a are mutated to alanine, this AAA mutant can no longer undergo phosphorylation and accumulates in the nucleus, allowing reconstitution of FOXO1a activity to PTEN null cells (Nakamura et al., 2000; Ramaswamy et al., 1999). Such reconstitution arrests cells in G1, inhibits soft-agar growth, and inhibits xenograft growth in nude mice, thus recapitulating many aspects of PTEN-mediated tumor suppression (Nakamura et al., 2000; Ramaswamy et al., 1999). Small molecules that block FOXO1a export could target members of the general protein transport machinery and inhibit proteins other than CRM1. In addition, small molecules may act as novel kinase inhibitors, revealing new aspects of FOXO1a signal transduction, and serve as preliminary anticancer therapeutics.

The present study describes a cell-based, chemical genetic screen using FOXO1a subcellular localization as the readout. Two classes of compounds that inhibit FOXO1a nuclear export were identified: (1) compounds that target the general nuclear transport machinery and (2) compounds specific to the PI3K/Akt/FOXO1a signaling pathway. Several compounds in the first class possess electrophilic moieties that most likely alkylate Cys528 in CRM1 by a manner similar to the mode of action of LMB. Compounds in the second class have been characterized further and implicate calmodulin as a mediator of FOXO1a nucleocytoplasmic localization and regulation.

Results

A cell-based screen for FOXO1a nuclear localization in PTEN null cells

In order to identify novel nuclear transport inhibitors as well as small molecules that target the PI3K/Akt/FOXO1a signaling pathway, a visual cell-based assay was developed that used FOXO1a subcellular localization as the output. PTEN null, 786-O renal carcinoma cells were infected with an adenovirus expressing FLAG epitope tagged FOXO1a (Ad-FKHR). Cells were grown for 24 hr after infection to allow for adequate expression of FLAG-FKHR and then treated with compounds for 1 hr, followed by immunostaining and imaging. In these cells, as previously published, FOXO1a is constitutively localized to the cytoplasm (Figure 1A). In contrast, FOXO1a localizes to both the nucleus and cytoplasm in $PTEN^{+/+}$ growing U2OS cells (Figure 1A). Treatment of infected cells with the PI3K inhibitor, wortmannin, led to FOXO1a relocalization to the nucleus and, likewise, treatment with the nuclear export inhibitor, LMB, also resulted in FOXO1a nuclear sequestration (Figure 1B). As a negative control, DMSO did not affect FOXO1a subcellular localization (Figure 1B). These data indicate that FOXO1a shuttles between the nucleus and cytoplasm in a PI3K- and CRM1-dependent manner and that the intracellular shuttling of FOXO1a is an appropriate measure of both PI3K/Akt pathway activation and CRM1 or possibly other nuclear export factor activation (Figure 1C).

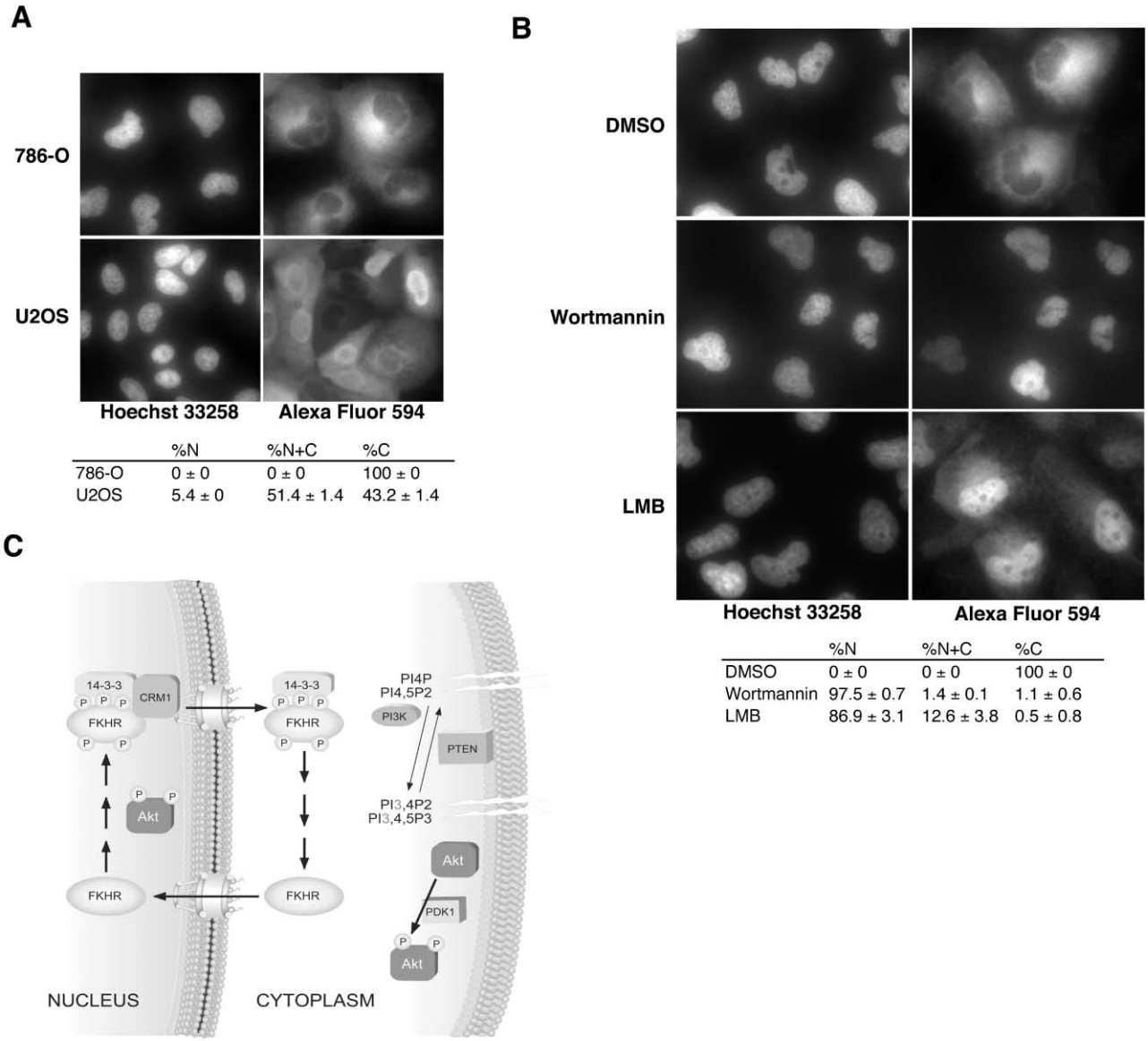
Using localization of FOXO1a as a visual assay, >18,000 compounds from the NCI Structural Diversity Set, ChemBridge DiverSetE, and a small collection of NCI marine extracts were tested for their ability to relocalize FOXO1a to the nucleus in PTEN null cells. Ad-FKHR-infected 786-O cells were grown on 384-well, clear-bottom plates before library compounds were transferred onto cells by a 384-pin array robot. Cells were fixed and stained after 1 hr treatment and FOXO1a localization determined by automated fluorescence microscopy. Eighty-nine compounds relocalized FOXO1a to the nucleus in this primary screen. Based on availability and potency, 42 total compounds were obtained for further characterization (seven from the NCI Structural Diversity Set, 34 from ChemBridge DiverSetE, and one from the NCI marine extracts).

Identification of general export inhibitors

In order to distinguish compounds that might target the general nuclear export machinery from those that target components of the PI3K/Akt/FOXO1a signaling pathway, each of the 42 small molecules identified in the primary screen was tested for the ability to block the export of HIV Rev, a protein known to undergo CRM1-dependent nucleocytoplasmic transport (Wolff et al., 1997). Here, compounds were serially diluted from 40 μM and added to U2OS cells stably expressing a RevGFP fusion protein containing the NES from PKI. As previously shown, this RevGFP fusion localizes to the cytoplasm at steady state (Figure 2A) (Henderson and Eleftheriou, 2000), and treatment of these cells with LMB leads to accumulation of RevGFP in the nucleolus (Figure 2A). Similarly, 19 of the initial 42 compounds blocked RevGFP export and are thus described hereforeward as "general export inhibitors" (Figure 2B).

The general export inhibitors target Cys528 of CRM1

LMB blocks NES-mediated nuclear export by covalently modifying CRM1 at Cys528 in humans and Cys529 in *S. pombe* by a Michael-type addition as illustrated in Figure 3A (Kudo et al., 1999, 1998). To determine whether any of these novel general export inhibitors similarly target the CRM1 Cys528 residue, U2OS-RevGFP cells were transfected with a dominant-negative CRM1 mutant expressing a Cys528Ser substitution. Cells expressing this mutant are viable and insensitive to LMB-mediated inhibition of nuclear export (Akakura et al., 2001) (Figure 3B). Furthermore, cells are still sensitive to LMB when overexpressing wild-type CRM1 (Akakura et al., 2001). Of the 19 general export inhibitors, 11 were inactive in CRM1-Cys528Ser transfected U2OS-RevGFP cells, suggesting that these compounds likely act by covalently modifying CRM1 at its reactive cysteine residue (Figure 3C). Approximately 50% of total cells exhibited cytoplasmic RevGFP—reflective of the transfection efficiency—in contrast to 100% nucleolar RevGFP in nontransfected cells. As a control, cells were also transfected with wild-type CRM1 and treated with the general export inhibitors. In these cells, the compounds blocked RevGFP export as they did in cells not overexpressing CRM1, as previously exhibited with LMB (data



	%N	%N+C	%C
786-O	0 ± 0	0 ± 0	100 ± 0
U2OS	5.4 ± 0	51.4 ± 1.4	43.2 ± 1.4

	%N	%N+C	%C
DMSO	0 ± 0	0 ± 0	100 ± 0
Wortmannin	97.5 ± 0.7	1.4 ± 0.1	1.1 ± 0.6
LMB	86.9 ± 3.1	12.6 ± 3.8	0.5 ± 0.8

Figure 1. FOXO1a subcellular localization and screening assay

A: PTEN$^{-/-}$ 786-O cells or PTEN$^{+/+}$ U2OS cells were infected with Ad-FKHR before FOXO1a cellular localization was visualized by immunofluorescence. In 786-O cells, FOXO1a is predominantly in the cytoplasm, whereas in U2OS cells, FOXO1a is both in the cytoplasm and nucleus. Nuclei were visualized by staining with Hoechst 33258. At least 200 cells were counted and the percent of predominantly nuclear (N), nuclear and cytoplasmic (N+C), and cytoplasmic (C) cells were determined with standard errors.

B: FOXO1a relocalizes to the nucleus in Ad-FKHR infected 786-O cells after treatment with wortmannin (39 nM) or LMB (4 nM), but not with DMSO.

C: Hyperphosphorylation of FOXO1a, as a result of PI3K/Akt signal transduction, promotes FOXO1a export into the cytoplasm. Inhibition of any one of the steps in the PI3K/Akt signaling pathway, as well as members of the nuclear export machinery, can lead to FOXO1a nuclear retention. PI3K phosphorylates PI4P or PI4,5P_2 and this reaction is reversed by the lipid phosphatase, PTEN. Upon PI3,4,5P_3 formation, Akt is recruited to the membrane and can undergo phosphorylation and activation by PDK1. FOXO1a is phosphorylated by an activated Akt, as well as other kinases, leading to binding with 14-3-3 and the promotion of nuclear export by CRM1.

not shown). The results for the remaining eight compounds were inconclusive in this assay. Thus, a second assay was developed in yeast to address the mode of action of these small molecules.

S. cerevisiae are insensitive to LMB because the yeast CRM1 contains a threonine at the homologous cysteine position (Neville and Rosbash, 1999). Cells can be made LMB sensitive by integration of a CRM1 mutant containing a Thr539Cys substitution in place of the wild-type yeast CRM1 (Neville and Rosbash, 1999). Based on these observations, wild-type yeast cells and cells containing the mutated CRM1 were transformed with a reporter plasmid expressing GFP containing both an NLS and an NES (Taura et al., 1998). In wild-type yeast cells, this protein shuttles between the nucleus and the cytoplasm, yet appears mostly in the yeast cytoplasm (Figure 3D). Wild-type yeast cells transformed with the NLS-NES-GFP reporter do not show accumulation of NLS-NES-GFP in the nucleus after treatment with LMB (Figure 3D). Conversely, in yeast cells expressing the CRM1 Thr539Cys mutation, shuttling of NLS-NES-GFP is

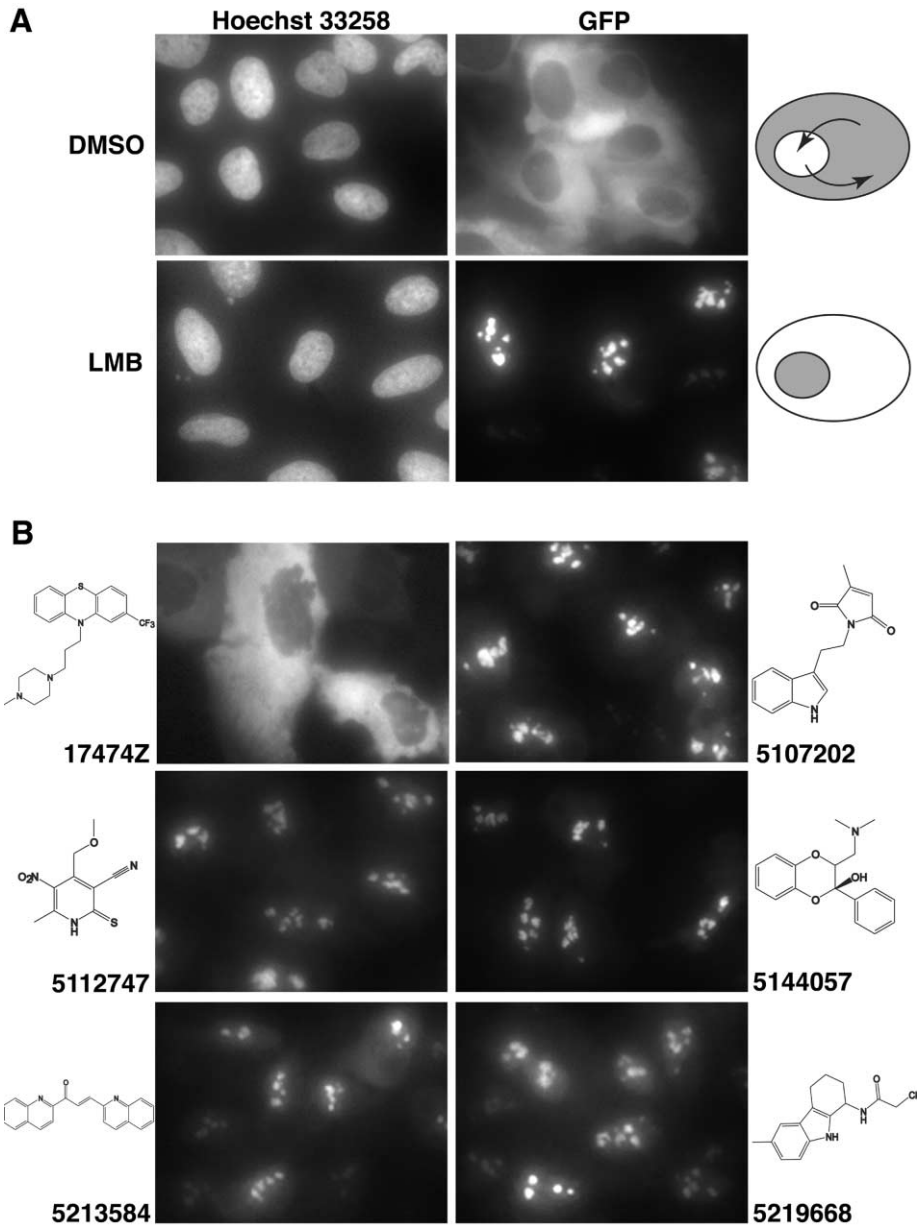


Figure 2. RevGFP export assay for general nuclear export inhibitors

A: U2OS cells stably expressing RevGFP were treated with DMSO or LMB. At steady state, RevGFP localizes to the cytoplasm in DMSO-treated cells while undergoing nucleocytoplasmic shuttling. However, in the presence of LMB (9.25 nM), RevGFP is trapped in the nucleoli due to a block in export in all cells. Nuclei were visualized by staining with Hoechst 33258.

B: U2OS-RevGFP cells were treated with all lead compounds resulting in 19 inhibitors of RevGFP export. Examples of five general export inhibitors at 20 μM trap RevGFP in the nucleoli of all cells (%N = 100) whereas the phenothiazine 17474Z at 20 μM does not (%N = 0).

blocked upon treatment with LMB. (Figure 3D). The 19 general export inhibitors, when retested in this assay, each blocked shuttling of NLS-NES-GFP in the humanized yeast. Thus, all appear to act through the CRM1 Cys528 residue (Figure 3E).

Compound structures reveal their mode of action

Comparing the structures of the 19 general export inhibitors to the structure of LMB revealed, in eight compounds, an α,β-unsaturated ketone or amide group that can likely undergo a Michael-type addition with the sulfhydryl group on Cys528 (Figure 4A; red). In addition, other compounds might undergo nucleophilic attack by the sulfhydryl group through a good halide leaving group (Figure 4A; blue), or across a triple bond (Figure 4A; green), or might undergo rearrangement for further reactivity (Figure 4A; pink). For three compounds, an obvious mechanism was not apparent though each was electrophilic (Figure 4A; black). Thus all of the general export inhibitors exhibit properties that likely explain the dependency of export inhibition on the CRM1 Cys528 residue.

Pathway-specific FOXO1a export inhibitors

Twenty-three compounds, though inhibitors of FOXO1a export, failed to alter export of RevGFP, and thus are termed pathway- or FOXO1a-specific. To determine whether such compounds were likely to act upstream or downstream of Akt, extracts were prepared from 786-O cells after treatment with each of the pathway-specific inhibitors or wortmannin, and phospho-Ser473-Akt was detected by immunoblotting. Two inhibitors (5219657 and B6-7-1) did not abolish phospho-Ser473-Akt levels similar to DMSO and thus likely have targets downstream of Akt or in a separate synergistic pathway (Figures 5A and 6). However, 21 compounds inhibited Ser473-Akt phosphorylation with varying

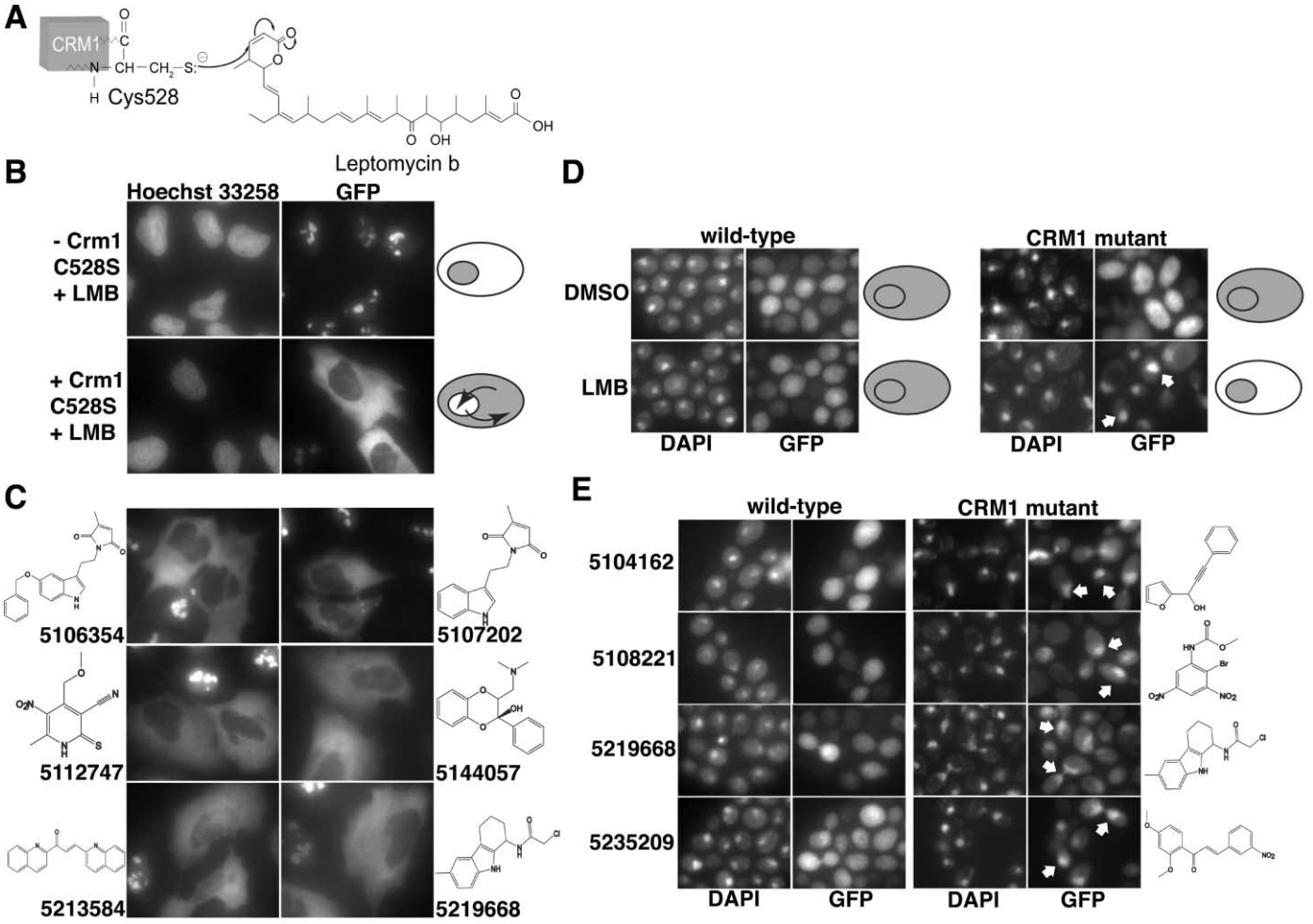


Figure 3. Assay for small molecules that target CRM1 in U2OS-RevGFP cells and yeast

A: LMB inhibits CRM1-mediated export by covalently modifying Cys528 by a Michael-type addition.

B: U2OS-RevGFP cells were either treated with 9.25 nM LMB or first transfected with the CRM1-Cys528Ser mutant before treatment with LMB. LMB blocks RevGFP export in nontransfected cells but does not block RevGFP export in cells expressing the CRM1-Cys528Ser mutant, resulting in about 50% cytoplasmic cells. Nuclei were visualized by Hoechst 33258 staining.

C: U2OS-RevGFP cells transfected with CRM1-Cys528Ser were treated with all 19 of the general export inhibitors. Here are examples of six compounds at 20 μM that do not block RevGFP export in cells expressing the CRM1 mutant, and thus target CRM1 at Cys528. About 50% of cells exhibited cytoplasmic RevGFP.

D: Wild-type yeast cells and humanized, mutant LMB-sensitive yeast cells were transformed with the NLS-NES-GFP reporter. NLS-NES-GFP is found throughout wild-type cells treated with either DMSO or 9.25 nM LMB. However, in CRM1 mutant, LMB-sensitive cells, 9.25 nM LMB treatment results in the nuclear accumulation of NLS-NES-GFP. DMSO treatment results in the reporter found throughout the cell. Nuclei were visualized by staining with DAPI.

E: All 19 of the general export inhibitors target CRM1 at the reactive cysteine residue when tested in wild-type and CRM1 mutant, LMB-sensitive yeast cells. Examples of various compounds (all at 20 μM) show NLS-NES-GFP localization throughout the cell in wild-type yeast and nuclear localization in CRM1 mutant cells. Arrows point to nuclear retention and nuclei were stained with DAPI for comparison.

efficacy. Phospho-Thr308 Akt was also probed and all compounds that blocked Ser473 phosphorylation similarly blocked Thr308 phosphorylation (Figures 5A and 6). As a control, treatment of cells with the general export inhibitors did not substantially alter Akt phosphorylation when tested at 20 μM, though three exhibited modest inhibition at 40 μM (Figure 6 and data not shown). Phospho-Ser255/Thr256 SGK levels were also probed revealing two compounds that significantly inhibited SGK phosphorylation (Figure 6). The two compounds that showed an effect, 5175309 and 5217339, might either exhibit less specificity or perhaps affect a parallel pathway leading to SGK and FOXO1a phosphorylation.

Akt nuclear localization was investigated in 786-O cells treated with pathway-specific inhibitors. If FOXO1a is phosphorylated by Akt in the nucleus, then it is possible that any compounds decreasing Akt phosphorylation levels and trapping FOXO1a in the nucleus might also result in the inhibition of FOXO1a phosphorylation by Akt mislocalization. Interestingly, treatment with the pathway-specific inhibitors did not affect Akt nuclear localization and overexpressed Akt as well as endogenous Akt was found throughout the cell (data not shown).

The pathway-specific compounds were next validated in a second assay in which FOXO1a localization is constitutively cytoplasmic based on expression of activated PI3K. Here, hu-

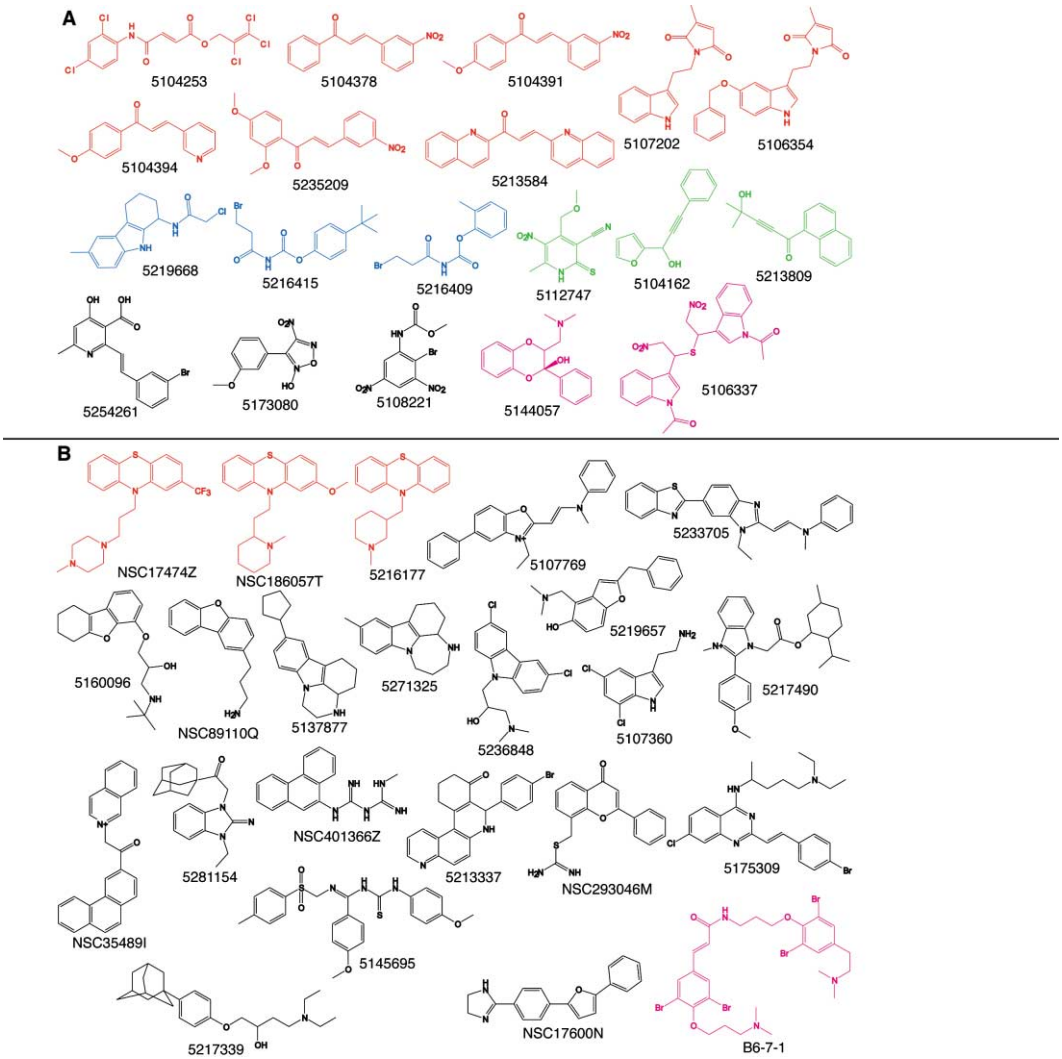


Figure 4. Structures of all lead compounds obtained for further characterization

A: General export inhibitors can covalently modify CRM1 like LMB either through an α,β-unsaturated ketone or amide as shown by compounds highlighted in red. Another method might be through the substitution of a good leaving group as shown by compounds highlighted in blue. Green highlighted compounds contain a triple bond, which may undergo nucleophilic addition. Compounds in pink might undergo rearrangement for further reactivity.
B: Structures of PI3K/Akt/FOXO1a specific inhibitors. Compounds in red belong to the structural family of phenothiazines. Several compounds contain planar heterocyles and may target the ATP binding sites of kinases. B6-7-1, in pink, was isolated from marine sponge extract.

man mammary epithelial cells (HMECs) containing either vector alone or stably expressing a membrane-targeted and, hence, constitutively active p110α subunit of PI3K (HMEC-Myr-p110α) (Zhao et al., 2003) were infected with Ad-FKHR. FOXO1a localization was then determined by immunofluorescence after treatment for 1 hr with each pathway-specific compound. In both cell types, FOXO1a is cytoplasmic. Thus the vector cells serve as a positive control for inhibition by a compound at any point along the PI3K pathway as well as a positive control for cell-specific drug metabolic properties such as cell permeability. Nine compounds that scored repeatedly in 786-O cells failed to score in the vector control cells, and thus their position in the pathway with respect to PI3K could not be formally established in this assay (Figures 5B and 6). Two compounds (89110Q, 293046M) blocked FOXO1a export in control cells, but not in HMEC-Myr-p110α cells, suggesting that these compounds have targets upstream of PI3K (Figures 5B and 6). Wortmannin and twelve pathway-specific inhibitors sequestered FOXO1a in the nucleus in both the vector and HMEC-Myr-p110α cells and thus likely act downstream of PI3K (Figures 5B and 6).

In order to determine whether structure-activity relationships existed with respect to these defined phenotypes, the structures of all pathway-specific inhibitors were examined. Several of these compounds contain planar heterocycles that might target the ATP binding sites of kinases. Examples include benzimidazoles (5217490, 5233705), an indole (5107360), a carbazole (5236848), hexahydrocarbazoles (5137877, 5271325), a quinazoline (5175309), a dihydrophenanthroline (5213337), a benzoxazole (5107769), benzofurans (5219657, 5160096), and a dibenzofuran (89110Q) (Figure 4B). B6-7-1, from a marine sponge extract, is an amino acid derived, novel bromotyrosine derivative (Figure 4B; pink).

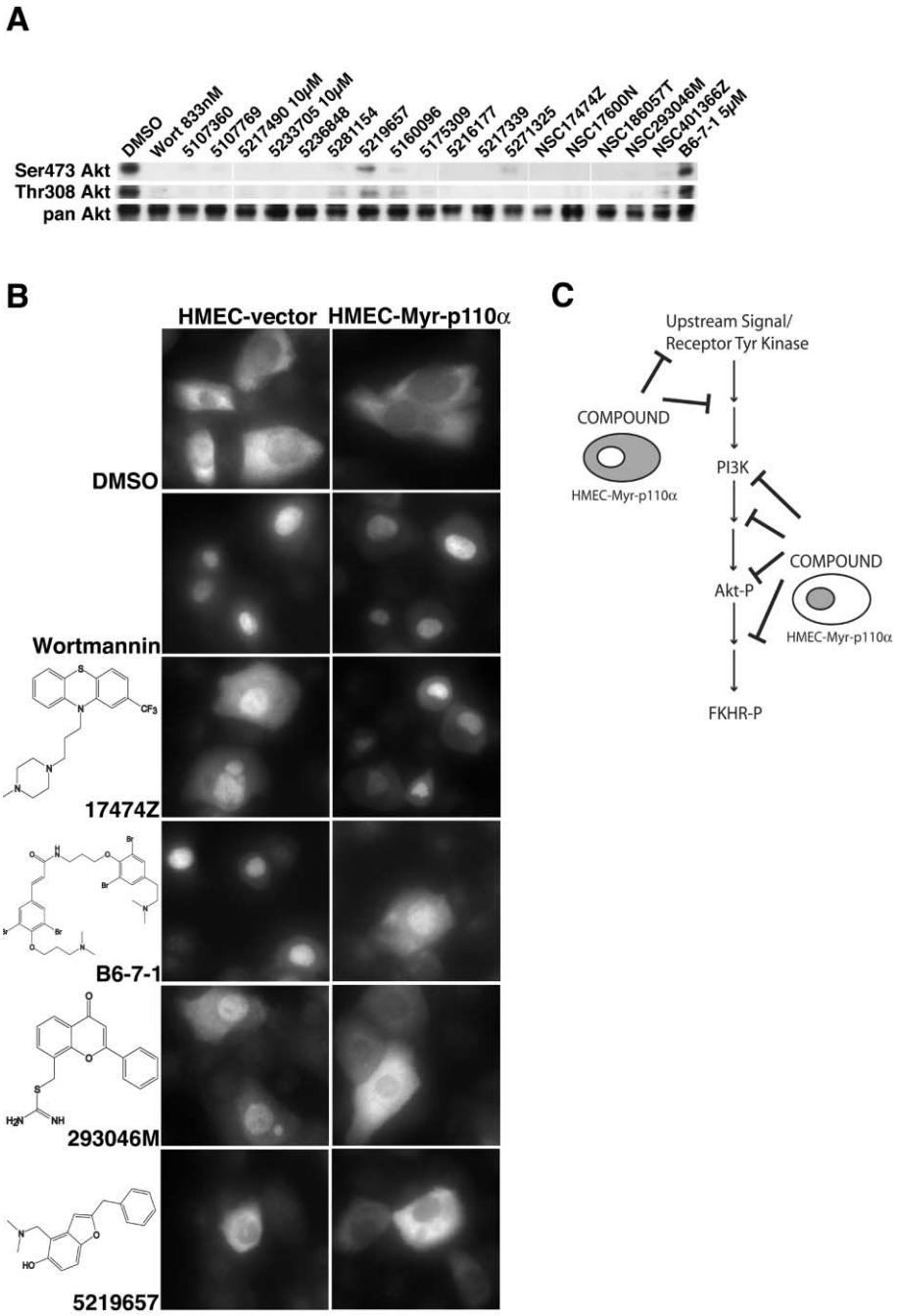


Figure 5. Most pathway-specific inhibitors decrease phospho-Ser473 and phospho-Thr308 Akt levels in 786-O cells, and block FKHR export in HMEC-Myr-p110α cells

A: Cell extracts were made from 786-O cells after treatment with pathway-specific compounds for 1 hr (all at 20 μM unless otherwise noted), and then immunoblotted with phospho-Akt and pan Akt antibodies.

B: FOXO1a localization in HMECs stably expressing Myr-p110α or vector control. HMECs were infected with Ad-FKHR and treated with pathway-specific compounds for 1 hr. Treatment with DMSO resulted in cytoplasmic FOXO1a in both HMEC-vector and HMEC-Myr-p110α cells. Treatment with 20 μM wortmannin, 40 μM trifluoperazine (17474Z), and 5 μM B6-7-1 results in nuclear FOXO1a in both cell types. Compound 293046M (80 μM) is an example of an inhibitor that causes FOXO1a nuclear localization in vector cells but not in Myr-p110α cells. Compound 5219657 (80 μM) is an example of an inhibitor that blocks FOXO1a export in neither cell types.

C: Compounds that trap FOXO1a in the nucleus in Myr-p110α cells target at or downstream of PI3K. Compounds that target upstream of PI3K block FOXO1a export in HMEC-vector cells but not in HMEC-Myr-p110α cells.

Phenothiazines—a class within the pathway-specific inhibitors

Of particular interest was the finding that three of the pathway-specific inhibitors belonged to the structural family of phenothiazines (Figure 4B; red) (17474Z, 186057T, 5216177), one of which was trifluoperazine (17474Z). Trifluoperazine, as well as many other phenothiazines, act as dopamine receptor antagonists and are clinically useful as antipsychotic and antiemetic medications. Like wortmannin, the three phenothiazines relocalized FOXO1a in 786-O cells to the nucleus, decreased phospho-Akt levels (Figure 5A), and in two instances blocked export in HMEC Myr-p110α cells (Figures 5B and 6).

To determine whether these activities were representative of the broader class of phenothiazines, additional phenothiazines including chlorpromazine, prochlorperazine, fluphenazine, and thioridazine were tested in the FOXO1a export assay. All of these compounds blocked FOXO1a export in 786-O cells (Figure 7A), raising the possibility that FOXO1a nuclear localization might be regulated by dopamine receptor signaling. Indeed, phospho-Akt levels increase when cells are treated with dopamine receptor agonists such as quinpirole and bromocriptine in neuronal cells (Brami-Cherrier et al., 2002; Kihara et al., 2002).

To investigate whether inhibiting the dopamine receptor can lead to FOXO1a nuclear retention, structurally unrelated dopamine receptor antagonists were tested in the FOXO1a export assay. None of the inhibitors—haloperidol, clozapine, L745870,

Compound	FOXO1a exp IC50 (μM)	REV exp IC50 (μM)	CRM1 C528S	CRM1 yeast	p-Akt S473	p-Akt T308	p-SGK S255	FOXO1a exp HMEC-vector	FOXO1a exp HMEC-p110α	Cell prolif. IC50 (μM)	Target
LMB	0.004	0.001								>40	CRM1
5104253	40	2.5								40	CRM1
5106354	20	2.5								5-10	CRM1
5107202	10	5								10-20	CRM1
5112747	10-20	5								20	CRM1
5144057	10-20	10								20	CRM1
5213584	20	2.5								2.5-5	CRM1
5216409	10-20	20								>40	CRM1
5216415	20-40	10								>40	CRM1
5219668	5	0.625								2.5-5	CRM1
5173080	10-20	20								20	CRM1
5254261	5-10	5								2.5-5	CRM1
5213809	20	10								2.5-5	CRM1
5104162	40	40								>40	CRM1
5104378	20-40	10								5	CRM1
5104391	20-40	10								5-10	CRM1
5104394	20	20								10-20	CRM1
5106337	10	2.5								2.5-5	CRM1
5108221	10	40								2.5-5	CRM1
5235209	20	10								5-10	CRM1
wortmannin	0.039									>40	PI3K
5107769	20									20	
5175309	20									5-10	
5217339	10-20									10-20	
5217490	5									2.5-5	
5233705	0.625									<1.25	
5236848	5									10	
NSC17474Z	20									20	CaM
NSC17600N	10									10	
NSC186057T	10									20-40	CaM
NSC35489I	5-10									20-40	
NSC293046M	10-20									40	
NSC89110Q	20									40	
5137877	40									10-20	
5160096	20-40									40	
5213337	10-20									40	
5216177	10-20									20-40	CaM
5271325	20-40									>40	
5281154	20									20-40	
NSC401366Z	10-20									20-40	
5107360	20									40	
5145695	40									20-40	
5219657	20									10	
B6-7-1	5	20-40								5-10	

inhibitory
weakly inhibitory
not inhibitory
not tested

Figure 6. Collective assay results for all lead compounds tested

Compound numbers beginning with "5" are the ID numbers from ChemBridge and those beginning with "NSC" are the ID numbers from the NCI. Dark gray denotes the compound scored in the assay (e.g., blocks FOXO1a or RevGFP export; inhibits CRM1 in U2OS-RevGFP transfected cells or in the yeast NLS-NES-GFP export assay; decreases phospho-protein levels; blocks cell proliferation). Light gray denotes the compound has weak activity in the assay and white denotes the compound showed no activity in the assay. Black denotes the compound was not tested in the assay.

or L-stepholidine (L-SPD)—significantly blocked FOXO1a export at concentrations comparable to those previously used to block dopamine receptors in cells (Dong et al., 1997; Patel et al., 1997; Vanhauwe et al., 2000) (Figure 7B). These data strongly suggest that the dopamine receptor is not the relevant target of the phenothiazines with respect to their activity as inhibitors of FOXO1a export.

Structurally unrelated CaM inhibitors block FOXO1a export

Trifluoperazine (17474Z), in addition to its dopamine receptor antagonist activity, also exhibits inhibitory activity against calmodulin (Levin and Weiss, 1976, 1977), raising the possibility that it and the phenothiazine class as a whole might interrupt PI3K signaling through calmodulin inhibition. To ask whether this was the case, Ad-FKHR infected 786-O cells were treated for 1 hr with the calmodulin inhibitors W-13, calmidazolium, and ophiobolin A. Each of these inhibitors relocalized FOXO1a to the nucleus (Figure 7C). Moreover, when tested in serial titrations, FOXO1a export was blocked at concentrations comparable to those previously reported for CaM inhibition by these compounds in cells (Mottet et al., 2003; Wei et al., 1983; Yang et al., 2000). Treatment with the Ca^{2+} chelator, BAPTA-AM, also blocked FOXO1a nuclear export at 80 μM (Figure 7C). However, treatment with EGTA, an extracellular calcium chelator, did not result in FOXO1a nuclear localization (data not shown). These data are consistent with the notion that CaM activity regulated by intracellular Ca^{2+} regulates FOXO1a subcellular localization.

786-O cell proliferation in presence of compounds

IC50s were determined for all 42 lead compounds in cell proliferation studies. In these studies, 786-O cells were grown in 96-well plates and treated for ~24 hr at decreasing concentrations of inhibitor serially diluted in complete media from 40 μM to 1.25 μM. Cell viability was determined by correlation with amount of ATP released when cells were lysed. The results for these experiments are reported in Figure 6, which also summarizes the results of the assays for all general export and pathway-specific inhibitors. Most of the inhibitors block cell proliferation; however, ten compounds exhibited no effect at 40 μM, including wortmannin. It is possible these compounds, as with wortmannin, are unstable in aqueous solution for an extended period of

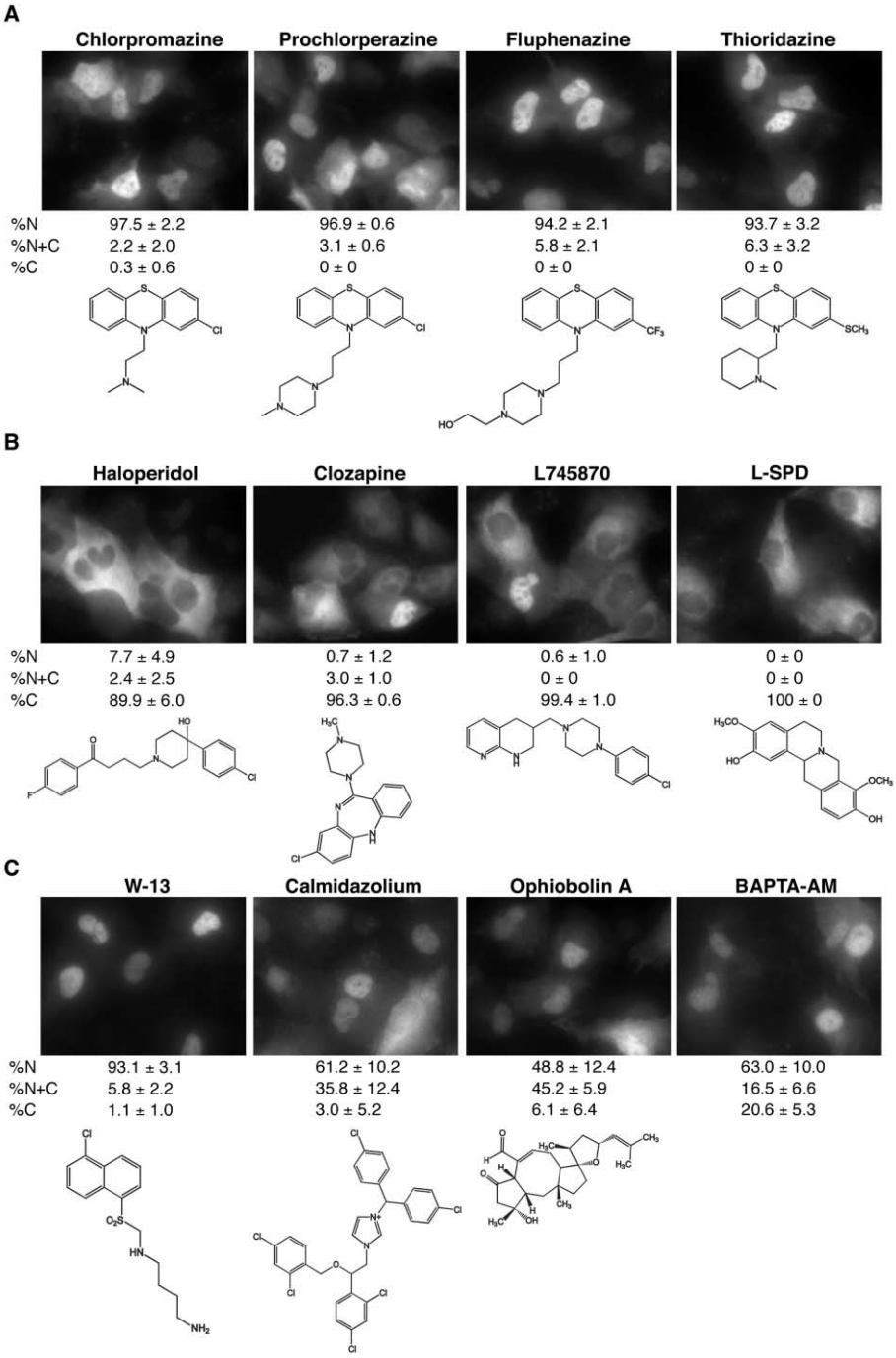


Figure 7. Trifluoperazine and other phenothiazines regulate FOXO1a subcellular localization through inhibition of CaM

A: 786-O cells infected with Ad-FKHR were treated with phenothiazine therapeutics. Chlorpromazine, prochlorperazine, fluphenazine, and thioridazine all relocalize FOXO1a to the nucleus at 20 μM.

B: Treatment with structurally unrelated dopamine receptor antagonists haloperidol (80 μM), clozapine (20 μM), L745870 (80 μM), and L-speridone (80 μM) do not inhibit FOXO1a export significantly.

C: Treatment with structurally diverse CaM inhibitors W-13 (40 μM), calmidazolium (20 μM), and ophiobolin A (5 μM) block FOXO1a export. BAPTA-AM, an intracellular Ca^{2+} chelator, inhibits FOXO1a export at 80 μM.

time (Stein and Waterfield, 2000; Woscholski et al., 1994). Thus, compounds that localize FOXO1a to the nucleus after 1 hr may not inhibit proliferation in this 24 hr assay.

Discussion

A cell-based, visual, chemical genetic screen for small molecule inhibitors of nuclear export of the transcription factor FOXO1a was carried out in cancer-derived cells lacking functional PTEN protein. The utility of this "phenotypic" screen was validated by the discovery of small molecules specifically targeting key cellular proteins, and as such, 19 novel CRM1 export inhibitors were identified. Furthermore, a calmodulin-dependent regulatory mechanism for controlling FOXO1a localization was discovered. These data suggest that such screens, when conducted in defined systems, can be highly informative and likely compliment traditional in vitro target-based drug screens.

We have shown that 19 of the compounds that promote retention of FOXO1a in the nucleus are novel general protein export inhibitors. All 19 block the nuclear export of RevGFP

and FOXO1a by targeting CRM1, and not by inhibition of other factors in the nuclear transport machinery. Interestingly, all of these general inhibitors were isolated from the ChemBridge DiverSetE and make up about half of the total hits from that library. While most, if not all, of the general blockers are reactive, not all of the reactive compounds in the library relocalized FOXO1a in this screen, suggesting that the compounds identified in the screen exhibit some selectivity for CRM1. Of the 19 compounds in this class, 5219668 is the most potent CRM1 inhibitor (Figure 6). While not as potent as LMB, in some cases this compound can make a suitable substitute when inhibition of general export is required in experiments.

Surprisingly, targeting of CRM1 by the general export inhibitors invariably required the Cys528 residue and the chemical structures suggest that this dependency likely results from covalent interactions. On the other hand, the Cys528 residue is not required for CRM1 function and the three-dimensional structure of CRM1 has not been determined. Therefore, the specific mechanism by which covalent attack on this residue leads to inhibition is not known. The diversity of this new expanded set of CRM1 inhibitors may shed light on this question. Specifically, while it appears that Cys528 binding is required, it may not be sufficient. Thus, determining the structure-activity requirements of the reactive compounds scoring in this assay may define any additional requirements.

Although *S. cerevisiae* express a form of CRM1 that does not contain the reactive cysteine residue, *S. pombe*, human, and most other organisms do. The subselection of specific reactive compounds interacting with CRM1 begs the question of whether the export receptor CRM1 can act as a general sensor for certain types of reactive compounds in nature. For example, cellular and transcriptional responses to small molecule toxins present in the environment might be mediated through CRM1 binding and inactivation. Such inactivation would render CRM1 unable to export NES-containing proteins and certain transcription factors, including FOXO proteins. Trapped in the nucleus, these proteins might then enact transcriptional programs resulting in a cell cycle arrest or apoptosis, as examples. In this light, CRM1 might act as a protector of the cell.

The utility of LMB as a therapeutic has been explored with respect to the role of CRM1 in regulating p53 localization. For example, trapping p53 in the nucleus with LMB results in its reactivation and transduction of its biological responses including cellular apoptosis (Hietanen et al., 2000; Lain et al., 1999a), leading some to propose targeting nuclear transport as a means of controlling cancer growth (Kau and Silver, 2003; Lain et al., 1999b). In phase I clinical trials, however, targeting CRM1 with LMB was associated with profound toxicity (Newlands et al., 1996). Cells expressing mutant CRM1 are resistant to LMB; thus, it is critical to understand whether patient-related toxicity is target related or due to non-CRM1, off-target effects of LMB. If patient-related toxicity is linked to non-CRM1 effects of LMB, then the possible lower toxicity of some of the compounds identified in this screen could potentially guide the preclinical development of CRM1 inhibitors with improved therapeutic index.

One of the inherent strengths of our FOXO1a-based screen is the ability to identify both general export inhibitors and more specific effectors of the signaling pathways that promote FOXO1a movement and activity. Thus, 23 small molecule inhibitors of FOXO1a export that appear to act in the PI3K/Akt/FOXO1a signaling pathway were identified. Among these were a novel natural product from marine sponge and several compounds containing planar aromatic heterocycles similar to the scaffolds of known kinase inhibitors. These latter compounds may potentially inhibit the activity of kinases involved in Akt signaling or FOXO1a phosphorylation and export. Experiments designed to localize the inhibitory activity of these compounds within the pathway are ongoing.

Three of the pathway-specific compounds belong to the structural family of phenothiazines, including trifluoperazine, a known dopamine receptor antagonist and CaM inhibitor. While trifluoperazine and several other phenothiazine dopamine receptor antagonists relocalized FOXO1a to the nucleus, structurally unrelated antagonists such as haloperidol, clozapine, L745280, and L-SPD did not. Trifluoperazine and other phenothiazines such as fluphenazine and chlorpromazine also inhibit CaM in addition to dopamine receptors. Additional structurally diverse calmodulin inhibitors such as W-13, calmidazolium, and ophiobolin A robustly relocalized FOXO1a to the nucleus. Furthermore, treatment of 786-O cells with the intracellular Ca^{2+} chelator, BAPTA-AM, likewise relocalized FOXO1a to the nucleus, strongly suggesting that calmodulin is a regulator of FOXO1a cellular location. Interestingly, the natural marine sponge product, B6-7-1, is a bromotyrosine and resembles the bastadins. Bastadins have been shown to modulate ryanodine receptors, Ca^{2+} release channels in the sarcoplasmic reticulum (Mack et al., 1994). Though untested, it is possible that B6-7-1 might block Ca^{2+} channels and thereby reduce the intracellular Ca^{2+} concentration. Such an effect, as mimicked by our experiment with BAPTA-AM, might prevent Ca^{2+} from binding to CaM, inactivate CaM, and therefore keep FOXO1a nuclear.

Reducing intracellular Ca^{2+} levels or inhibiting CaM leads to inactivation of Akt in PC12 cells (Egea et al., 2001). Similarly, BDNF activation of Akt is blocked in cells expressing CaM with mutant Ca^{2+} binding domains (Cheng et al., 2003). The calmodulin inhibitor, W-13, while unable to alter PI3K or Akt kinase activity in vitro, decreases phospho-Akt levels in adipocytes in a PI3K-independent manner (Egea et al., 2001; Yang et al., 2000). Together, these suggest the presence of a CaM-dependent, PI3K-independent path to Akt activation. Finally, Ca^{2+}/calmodulin-dependent protein kinase kinase (CaM-KK) can phosphorylate Akt and SGK1 through a Ca^{2+}/CaM signaling pathway (Imai et al., 2003; Yano et al., 1998). Thus, one possibility is that FOXO1a cellular localization and activity might be regulated by a Ca^{2+}/CaM signaling cascade that results in the activation of CaM-KK and phosphorylation of both Akt and SGK1 by CaM-KK.

A schematic of the pathway depicting the site of action for the compounds that we have characterized thus far is shown in Figure 8. Several of the compounds might prove to be leads for potential anticancer drug development including trifluoperazine and 5233705. There is an ongoing vigorous debate on the virtues or perils of cell-based screening. This particular screen proved robust, facile, highly amenable to academic scale screens, and more importantly, elucidated previously undiscovered export and PI3K pathway inhibitors. Furthermore, we have determined the specific molecular mechanism for inhibition of representative compounds within both classes of inhibitors, leading to the discovery of novel CRM1 inhibitors and revealing the role of CaM in regulating PI3K signaling to FOXO1a. Experi-

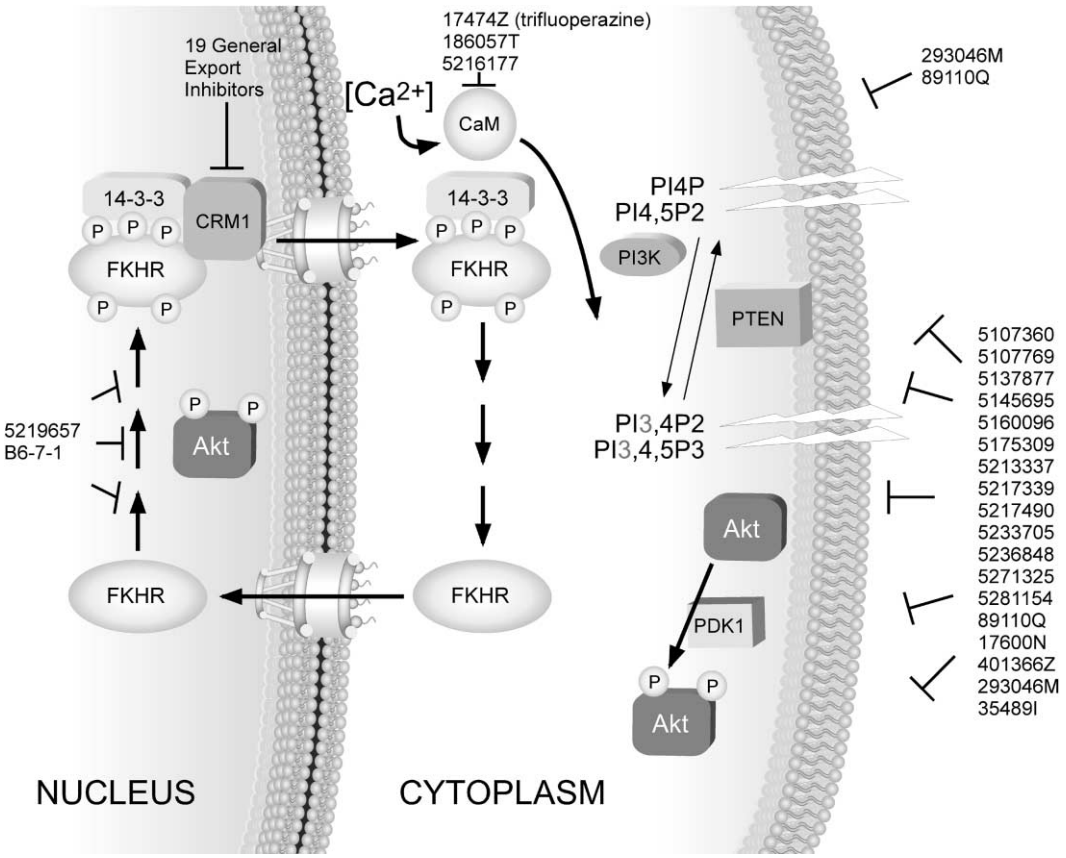


Figure 8. Target sites of compounds identified from the screen

Two inhibitors have targets located upstream of PI3K, 16 compounds target somewhere between PI3K and Akt inclusively, two inhibitors have targets downstream of Akt, 19 inhibitors target CRM1, and three inhibitors target CaM and implicate CaM as a novel regulator of FOXO1a activity and subcellular localization.

ments are currently underway to identify the targets of the remaining pathway-specific compounds.

Experimental procedures

Materials

LMB, wortmannin, and haloperidol were purchased from Sigma. L-SPD, W-13, calmidazolium, and ophiobolin A were purchased from Calbiochem. BAPTA-AM was purchased from Molecular Probes. L754870 was purchased from Tocris. Clozapine was purchased from Alexis Corp. Phenothiazine therapeutics thioridazine, fluphenazine, chlorpromazine, and prochlorperazine were purchased from ICN Biomedicals, Inc.

Lead compounds from the screen were ordered from ChemBridge Corp. or requested from the NCI. The pRev(1.4)-GFP+PKI NES plasmid was a gift from Beric Henderson and its construction described in Henderson and Eleftheriou (2000). The CRM1-Cys528Ser mutant plasmid, pXHCK1, was a gift from Minoru Yoshida and previously described in Akakura et al. (2001). The NLS-NES-GFP plasmid (pPS1372) was previously described in Taura et al. (1998).

Cell culture and yeast strains

786-O and U2OS-RevGFP cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Clone (Clonetech) and 100 μg/ml penicillin-streptomycin at 37°C, 5% CO_2. U2OS-RevGFP cells were established by transfecting U2OS cells with pRev(1.4)-GFP+PKI NES using FuGENE 6 reagent according to the manufacturer's protocol (Boehinger Mannheim). Stable clones were selected in complete media containing 400 μg/ml G418. Human mammary eptithelial cells, HMEC-Myr-p110α and HMEC-vector, were previously described in Zhao et al. (2003). Cells were grown in mammary epithelial basal medium (MEBM, BioWhittaker) supplemented with mammary epithelial growth medium (MEGM, BioWhittaker) consisting of hydrocortisone, EGF, insulin, and bovine pituitary extract.

Wild-type yeast PSY580 and CRM1 mutant yeast PSY1969 were transformed with the NLS-NES-GFP plasmid using a standard transformation protocol. Transformants were selected on ura^- dropout plates.

Adenovirus construction

Ad-FKHR was generated with the pAD-Easy system (He et al., 1998). In brief, linearized shuttle plasmid containing the cDNA for FLAG-FOXO1a was cotransfected with pAdEasy-1 into BJ5183 cells. After isolation, recombinant adenoviral DNA was restriction digested with PacI and transfected into 293 cells. Infectious adenovirus was amplified in 293 cells. Purified virus was isolated by freeze-thaw extraction followed by CsCl gradient purification and titred by plaque lysis.

FOXO1a export assay in 786-O and HMEC cells

786-O cells were seeded onto 384-well, black, clear-bottom plates (Costar) at a density of ~2500–3000 cells/well in 50 μl complete media. After incubation at 37°C, 5% CO_2 for 2–3 hr, cells were infected with Ad-FKHR and incubated for ~24 hr. Small molecule compounds were serially diluted 1:2 starting from 80 μM in a separate 384-well plate using a 16-channel pipette (ThermoLabsystems) in DMEM. Media were aspirated from infected cells using a 24-channel wand before diluted small molecules were transferred onto cells. Cells were incubated for ~1 hr before 3.7% formaldehyde fixation. Fixed cells were then stained with M5 anti-FLAG antibody (Sigma) followed by washing with PBS three times and staining with Alexa Fluor 594 goat

anti-mouse antibody (Molecular Probes) and Hoechst 33258 (Sigma). Both antibodies, M5 and Alexa Fluor 594, were diluted 1:1000 in PBS/0.2%TX-100/5%FBS. For cell counts, at least 200 cells exhibiting nuclear, nuclear and cytoplasmic, or cytoplasmic staining were counted from three separate images. Percentages of N, N+C, and C cells were calculated and standard deviations determined.

HMEC's were treated similarly except they were seeded at a density of ~3500 cells/well and incubated overnight before infection with Ad-FKHR virus. Compounds were diluted in MEBM supplemented with MEGM.

High-throughput screen

Cell-based screening was performed at the Institute for Chemistry and Cell Biology (http://iccb.med.harvard.edu). 786-O cells were seeded onto black, clear-bottom, 384-well plates at ~2500–3000 cells/well in 50 μl complete media using a Multidrop liquid dispenser (Labsystems). Cells were then incubated for 3 or 17 hr before infection with Ad-FKHR for ~24 hr. Compounds from the ChemBridge DiverSetE (ChemBridge Corp.) and the NCI Structural Diversity Set (NCI) were kept at a stock concentration of 5 mg/ml (~10 mM) in DMSO. 100 nl of each compound, as well as those from the NCI marine extract plate, were transferred to cells by a solid 384-pin array device attached to a robotic arm. Cells were then incubated for ~1 hr at 37°C, 5% CO_2. Formaldehyde fixation and antibody staining were performed as described in the FOXO1a export assay.

Digital images of cells in each well were acquired using an automated fluorescence microscope equipped with a Plan Fluor 10X NA 0.3 objective (Nikon) and Metamorph software (Universal Imaging). FOXO1a subcellular localization was scanned visually for each imaged plate.

RevGFP export and CRM1 target assays

U2OS-RevGFP cells were seeded onto clear-bottom, black, 384-well plates at ~4500 cells/well in 50 μl complete media. Cells were allowed to attach and grow overnight before compound treatment. Compounds were serially diluted 1:2 starting from 40 μM in a separate 384-well plate in DMEM. Media were aspirated from cells before the diluted compounds were transferred onto cells. Cells were incubated with compound for ~1 hr before fixation with 3.7% formaldehyde and nuclei staining with Hoechst 33258. For the CRM1 target assay, U2OS-RevGFP cells were transfected with pXHCK1, the CRM1-Cys528Ser mutant, on a 10 cm plate using FuGENE 6 transfection reagent according to the manufacturer's directions. After ~17–24 hr of incubation, cells were detached and re-plated onto a black, clear-bottom, 384-well plate at ~4500 cells/well density. Cells were allowed to flatten and grow overnight before treatment with various compounds for ~1 hr. Fixing and staining procedures were similar to those used in the RevGFP export assay.

Yeast CRM1 assay

Wild-type PSY580 and CRM1 mutant PSY1969 yeast cells expressing the NLS-NES-GFP reporter (pPS1372) were grown to log phase in 10 ml YEPD media. Small molecule inhibitors were diluted in YEPD to a final concentration of 20 μM. 200 μl of cells were then aliquoted into individual wells of a 96-well, U-bottom plate. Cells were pelleted and resuspended in media containing diluted inhibitors and treated for ~1 hr at 30°C before fixation in 14% formaldehyde. Fixed cells were then washed twice and resuspended in 100 μl of Solution P (0.1 M potassium phosphate buffer [pH 6.5], 1.2 M sorbitol). 20 μl of cells was pipetted into each well of a black, polylysine-treated, 24-well slide. Cells were permeabilized with 0.5% NP-40 or Triton X-100 in Solution P before nuclei staining with 1 μg/ml DAPI in Aby Wash 2 (0.1 M Tris [pH 9.5], 0.1 M NaCl, 50 mM $MgCl_2$). Coverslips were then mounted onto slides and cells visualized using an inverted fluorescence microscope with a 60× objective. Digital images were acquired using Metamorph software.

Phospho-Akt and phospho-SGK immunoblot

786-O cells were grown to confluency in 6-well tissue culture plates. Cells were then treated with compound diluted in 2 ml DMEM at decreasing concentrations, (e.g., 40, 20, and 10 μM) for ~1 hr at 37°C, 5% CO_2. Cells were then scraped in PBS, pelleted, and lysed in extract buffer (10 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.5% deoxycholate, 1 mM PMSF, PLAC). Lysates were spun at 14,000 × g for 10 min and supernatants aliquoted, frozen on dry ice, and stored at −80°C. Protein concentrations were determined using Bradford reagent (Bio-Rad) and aliquots of lysates with equal amounts of protein were resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore). Membranes were blocked in 5% powdered milk in PBST (PBS, 0.2% Tween-20), incubated overnight at 4°C in phospho-Ser473-Akt antibody (Cell Signaling) diluted 1:1000 in PBST containing 5% BSA. Phospho-Ser473-Akt levels were detected using anti-rabbit HRP diluted 1:5000 in 5% milk/PBST followed by Western Lightning chemiluminescence reagent (PerkinElmer). Blots were stripped in stripping buffer (100 mM glycine, HCl/pH 2.5), washed twice in PBST, and blocked in PBST containing 5% milk before re-probing with pan Akt antibody (Cell Signaling) diluted 1:1000 in PBST/5% BSA. Phospho-SGK (Upstate) was diluted 1:200 in PBST/5%BSA. For phospho-Thr308-Akt (Cell Signaling) immunoblots, membranes were blocked in 4% milk in TBST and secondary anti-rabbit HRP was diluted 1:3000 in 2% milk/TBST.

Cell viability assay

786-O cells were seeded onto opaque, white, 96-well plates (Costar) at ~2500 cells/well density and incubated for 24 hr. Compounds were serially diluted 1:2 from 40 μM to 1.125 μM on a different 96-well plate in serum-containing media. 100 μl of each dilution was transferred onto cells after removal of media. Cells were incubated with inhibitors for ~24 hr. Cell viability was assayed using CellTiter-Glo luminescent cell viablilty assay (Promega) following the manufacturer's protocol.

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The Psammaplysenes, Specific Inhibitors of FOXO1a Nuclear Export

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A small collection of marine natural product extracts was screened for compounds that would compensate lost tumor suppressor functionality in PTEN-deficient cells. From the most active extract, the previously unreported bromotyrosine derivative, psammaplysene A (**1**), was identified. Psammaplysene A compensates for PTEN loss by relocalizing the transcription factor FOXO1a to the nucleus.

Cancer cells have gain-of-function or loss-of-function mutations, or both, that lead to unchecked cell proliferation. Small molecules can modulate gain-of-function mutations by inhibiting the mutated gene product,[1] but small molecule modulation of loss-of-function mutations has been quite difficult. Finding targets downstream of the loss-of-function mutation, which are amenable to small molecule modulation, is likely to be a more productive, if still unproven, approach. This paper describes the discovery of two previously unreported natural products from the marine sponge *Psammaplysilla* sp. These compounds were identified from a high-content screen for small molecules that restore the function of FOXO1a, a downstream target of the PTEN tumor suppressor.[2]

The path linking PTEN with FOXO1a involves several steps, not all of which are well understood (Figure 1). Loss of PTEN phosphatase activity has been noted in Cowden's disease, a hereditary disease with a marked predisposition for breast and thyroid cancers, and PTEN phosphatase deficiencies have been observed in many other cancers.[3] FOXO1a, a member of the Forkhead family of transcription factors, which negatively regulates cell cycle progression and cell survival, is an attractive downstream target for small molecule modulation of loss of PTEN function. As a result of loss of PTEN phosphatase activity, phosphorylated FOXO1a remains inappropriately localized in the cytoplasm and unable to restrain cell cycle progression.[4] Small molecules that would enforce the nuclear re-localization of FOXO1a would be, at a minimum, useful tools to investigate FOXO1a regulation and cell growth.

A cell-based screen to identify such small molecules used the subcellular localization of FOXO1a as a readout, and libraries of ~18 000 synthetic molecules (NCI Structural Diversity Set, Chembridge DiverSetE) and 352 uncharacterized extracts from the NCI collection were assayed in this primary screen.[2] Among the strongest screening positives was a dichloromethane/methanol extract from a marine sponge, *Psammaplysilla* sp., which was collected in the Indian Ocean.

For isolation of the active component, 200 mg of this extract was subjected to activity-guided fractionation. A simplified Kupchan solvent-partitioning scheme yielded hexane, dichloromethane, and methanol/water fractions, of which the dichloromethane fraction was most active. Further fractionation via silica gel chromatography followed by reversed-phase HPLC yielded two pure active compounds, which were named psammaplysene A (**1**, 7.2 mg) and psammaplysene B (**2**, 0.7 mg). Positive-ion electrospray ionization MS indicated formula weights of 765 and 751, respectively, whereby the isotopic pattern suggested the presence of four bromine atoms in both compounds. High-resolution electrospray MS gave $C_{27}H_{35}Br_4N_3O_3$ as the molecular formula for psammaplysene A. Further structural characterization using a standard set of 2D NMR experiments including dqf-COSY, NOESY, HMQC, and HMBC was straightforward. Psammaplysene A (**1**) and psammaplysene B (**2**) were characterized as previously unreported dimeric bromotyrosine alkaloids (Chart 1), each consisting of two modified dibromotyrosine units combined with fragments most likely derived from aliphatic amino acids.

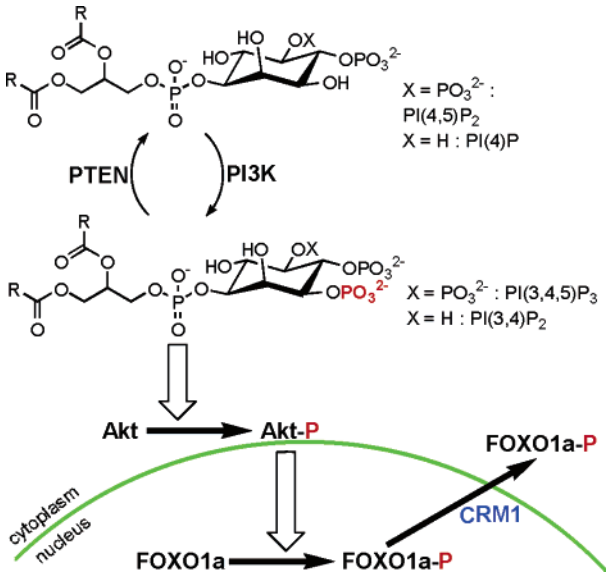

Figure 1. PTEN/PI3K/Akt/FOXO1a signaling pathway.[2] Akt phosphorylates FOXO1a, inducing FOXO1a nuclear export via CRM1. PTEN counteracts the kinase PI3K by dephosphorylating the lipid phosphates $PI(3,4)P_2$ and $PI(3,4,5)P_3$, thereby down-regulating phosphorylation of Akt and thus FOXO1a. In PTEN-null cells, FOXO1a remains constitutively phosphorylated and localized in the cytoplasm, which leads to cellular proliferation.

Psammaplysene A was among the most active inhibitors in this assay ($IC_{50} = 5\ \mu M$), whereas psammaplysene B was somewhat less active ($IC_{50} = 20\ \mu M$) (Figure 2). Among all of the compounds screened (>18 000), only five have IC_{50} values $\leq 5\ \mu M$.[2] Interestingly, the known dimeric bromotyrosine purpuramine-1 (**3**),[5] which was among the minor

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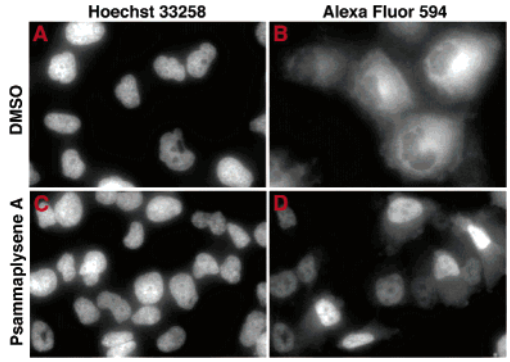

Figure 2. FOXO1a nuclear export inhibition by psammaplysene A (**1**). (A) PTEN-deficient cells[2] treated with DMSO (control) and stained with Hoechst 33258 to visualize nuclei and (B) stained with Alexa Fluor 594 to visualize localization of FOXO1a. Here, FOXO1a is predominantly in the cytoplasm. (C) cells treated with 5 μM psammaplysene A in DMSO stained with Hoechst 33258 and (D) stained with Alexa Fluor 594, showing re-localization of FOXO1a to the nuclei.

Chart 1. Structures of Psammaplysenes A (**1**) and B (**2**), Purpuramine-1 (**3**), Bastadin-5 (**4**), and Psammaplin A (**5**)

components in the extract, was not active in our assay. Purpuramine-1 and several homologous compounds, originally isolated from *Psammaplysilla purpurea*, were shown to have antibacterial properties.[6]

Sponges of the order Verongida to which the genus *Psammaplysilla* belongs are known to produce a wide range of structurally diverse bromotyrosine derivatives,[7] most prominent among them the sulfide-bridged psammaplins, such as psammaplin A (**5**), a histone deacetylase inhibitor,[8] and the macrocyclic bastadins, for example bastadin-5 (**4**),[9] a potent agonist of the RyR1 calcium channel.[10] The psammaplysenes (**1**, **2**) differ from related structures such as purpuramine-1 (**3**) and bastadin-5 (**4**) by having an α,β-unsaturated amide linkage and are distinguished by the way the two bromotyrosine subunits are connected.

There are multiple mechanisms to keep FOXO1a in the nucleus, and a series of secondary assays was used to assign the screening positives (42 including psammaplysene A) to distinct mechanistic classes. CRM1 is a general nuclear export receptor whose inhibition would localize FOXO1a and many other proteins in the nucleus.[11] A screen to assess whether compounds targeted CRM1 identified roughly half of the screening positives, 19 compounds, as CRM1 inhibitors and thus not specific inhibitors of the PI3K/Akt/FOXO1a signaling pathway. Psammaplysene A was not active in this secondary assay at 5 μM and thus considered pathway specific.[2]

Compounds specific for the PI3K/Akt/FOXO1a pathway could have targets upstream or downstream of Akt (Figure 1). By measuring levels of phosphorylated Akt by immunoblotting, the 23 pathway-specific compounds were assigned to these two classes. A total of 21 compounds led to decreased Akt phosphorylation, and two, including psammaplysene A, led to no change in Akt phosphorylation compared to untreated controls.[2] Psammaplysene A must have a target, which is as yet unidentified, downstream of Akt.

Among the five most potent positives in our primary screen, psammaplysene A (**1**) is the only compound that neither inhibited CRM1 nor reduced Akt phosphorylation. The target of psammaplysene A is not known, but small alterations of the basic structure, the removal of a methyl group to give psammaplysene B (**2**) for example, significantly diminish activity. The discovery of psammaplysene A's activity in this set of assays demonstrates that crude natural product extracts can be used to find potent and specific inhibitors in high-content, cell-based assays such as the one described. The highly modular psammaplysenes contain several easily accessible subunits and should therefore be amenable to synthesis-based exploration of structure−activity relations.

Experimental Section

General Experimental Procedures. NMR spectra were recorded at 25 °C using Varian INOVA500 (500 MHz proton, 126 MHz carbon) and Varian INOVA600 (600 MHz proton, 151 MHz carbon) spectrometers with CD_3OD or CD_2Cl_2 as the solvent. Double quantum filtered COSY (DQF-COSY) spectra were acquired using the standard pulse sequences and phase cycling. Phase-sensitive NOESY spectra were acquired with a mixing time of 600 ms. HMQC spectra were acquired in the phase-sensitive mode without gradients using phase-cycling for coherence selection. In some cases additional gradient HMQC and HSQC spectra were acquired. Magnitude-mode HMBC spectra were acquired without gradients and using phase-cycling for coherence selection. HMQC and HMBC spectra for psammaplysene B were acquired using Shigemi NMR tubes. Mass spectra were acquired using a Micromass Quattro II (positive-ion electrospray ionization), while high-resolution MS were obtained on a Micromass Autospec (positive-ion electrospray ionization). HPLC employed an Agilent 1100 series HPLC system with diode-array detector (190−900 nm) using a Supleco Discovery HS C-18 column (25 cm × 10 mm, 5 μm particle diameter).

Isolation of Psammaplysene A (1) and Psammaplysene B (2) by Activity-Guided Fractionation. Marine extract NCI-C013823-F3 (200 mg) was dissolved in 10 mL of a 9:1 mixture of methanol and water. The solution was extracted with two 10 mL portions of hexanes. Subsequently, the water content of the methanol phase was adjusted to 33%, followed by extraction with two 5 mL portions of dichloromethane. The resulting hexanes, dichloromethane, and methanol/water fractions were concentrated in vacuo and tested in

Table 1. ^{1}H and ^{13}C NMR Data of Psammaplysene A (**1**) (solvent CD_3OD, spectra referenced to 3.31 ppm for CD_2HOD, and 49.05 ppm for CD_3OD, coupling constants in Hz)

position	δ C	δ H	relevant HMBC correlations
1	56.6	3.48 (t, $J = 7$)	C-26/27
2	26.2	2.32 (quintet, $J = 7$)	
3	71.2	4.16 (t, $J = 7$)	C-4
4	154.24		
5	119.26		
6	132.8	7.82	C-4, C-10
7	135.8		
8	132.8	7.82	C-4, C-10
9	119.3		
10	137.6	7.39 ($J = 15.7$ Hz)	C-6/8, C-7, C-12
11	124.1	6.64 ($J = 15.7$ Hz)	C-12
12	167.5		
N−H		6.6 (br. t)	
13	36.6	3.60 (t, $J = 7$)	C-12
14	30.5	2.13 (quintet, $J = 7$)	
15	71.7	4.07 (t, $J = 7$)	C-16
16	153.2		
17	119.1		
18	134.1	7.59	C-16, C-19
19	136.8		
20	134.1	7.59	C-16, C-19
21	119.1		
22	30.3	3.00 (t, $J = 7$)	C-18/20, C-19
23	59.0	3.27 (t, $J = 7$)	C-19, C-24/25
24, 25	43.3	2.87 (s)	C-23, C-24/25
26, 27	43.4	2.94 (s)	C-1, C-26/27

our assay, which showed the dichloromethane fraction to be most active. For further purification, the dichloromethane fraction was chromatographed over silica using dichloromethane/methanol mixtures containing 0.4% concentrated aqueous ammonia as the solvent. Starting with 10% methanol, the methanol content of the solvent was gradually increase to 40%. Fractions eluting between 20 and 30% methanol were active and thus combined, concentrated, and subsequently rechromatographed using the same solvent system. This separation yielded two distinct fractions containing bromotyrosine-derived alkaloids. The earlier-eluting fraction contained almost pure purpuramine-1 (**3**), while the second fraction contained a mixture of psammplysenes A and B. The mixture of the two psammaplysenes was separated by reversed-phase HPLC using methanol/water mixtures with methanol contents of 35−100% as solvent, which yielded 7.2 mg of psammaplysene A (**1**, purity by NMR > 98%) and 700 μg of psammaplysene B (**2**, purity by NMR > 90%).

Psammaplysene A (1): ^{1}H and ^{13}C NMR data, see Table 1; positive-ion ESIMS *m/z* 775 (4), 774 (15), 773 (15), 772 (60), 771 (25), 770 (87), 769 (16), 768 (56), 767 (5), 766 (14) (ion cluster corresponding to M + H^+), 387.5 (20), 386.5 (68), 385.5 (100), 384.5 (70), 383.5 (18) (ion cluster corresponding to M + $2H^+$); positive-ion HRESIMS *m/z* 767.9443 (calcd for $C_{27}H_{36}{}^{79}Br_3{}^{80}BrN_3O_3$ 767.9470).

Psammaplysene B (2): ^{1}H and ^{13}C NMR data, see Table 2; positive-ion ESIMS *m/z* 761 (3), 760 (13), 759 (14), 758 (56), 757 (20), 756 (80), 765 (14), 754 (53), 753 (5), 752 (12) (ion cluster corresponding to M + H^+), 380.5 (19), 379.5 (69), 378.5 (100), 377.5 (69), 376.5 (17) (ion cluster corresponding to M + $2H^+$); positive-ion HRESIMS *m/z* 753.9247 (calcd for $C_{26}H_{34}{}^{79}Br_3{}^{80}BrN_3O_3$ 753.9314).

Table 2. ^{1}H and ^{13}C NMR Data of Psammaplysene B (**2**) (solvent CD_3OD, spectra referenced to 3.31 ppm for CD_2HOD, and 49.05 ppm for CD_3OD, coupling constants in Hz)

position	δ C	δ H	relevant HMBC correlations
1	49.0	2.83 (t, $J = 7$)	C-26
2	30.2	2.02 (quintet, $J = 7$)	
3	72.5	4.07 (t, $J = 7$)	C-4
4	154.1		
5	118.8		
6	131.9	7.66	C-4, C-10
7	133.9		
8	131.8	7.66	C-4, C-10
9	118.8		
10	137.0	7.40 ($J = 16$ Hz)	C-6/8, C-12
11	123.2	6.36 ($J = 16$ Hz)	C-12
12	164.8		
N-H		6.31 (br t)	
13	38.0	3.67 (t, $J = 7$)	C-12
14	29.8	2.10 (quintet, $J = 7$)	
15	72.3	4.10 (t, $J = 7$)	C-16
16	151.1		
17	117.9		
18	133.1	7.39	C-16, C-19
19	140.31		
20	133.1	7.39	C-16, C-19
21	117.9		
22	33.0	2.68 (t, $J = 7$)	C-18/20
23	60.8	2.47 (t, $J = 7$)	C-19, C-24/25
24, 25	45.2	2.21 (s)	C-23, C-24/25
26	36.2	2.43 (s)	C-1

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Supporting Information Available: ^{1}H NMR, DQF-COSY, coupled gHSQC, and HMBC spectra of psammaplysene A, as well as ^{1}H NMR, DQF-COSY, NOESY, HMQC, and HMBC spectra of psammaplysene B.

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Akt-regulated pathways in prostate cancer

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Prostate cancer remains a major cause of cancer-related mortality. Genetic clues to the molecular pathways driving the most aggressive forms of prostate cancer have been limited. Genetic inactivation of PTEN through either gene deletion or point mutation is reasonably common in metastatic prostate cancer and the resulting activation of phosphoinostide 3-kinase, AKT and mTOR provides a major therapeutic opportunity in this disease as mTOR inhibitors, HSP90 inhibitors and PI3K inhibitors begin to enter clinical development.
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Keywords: PTEN; AKT; prostate cancer; phosphoinostide 3-kinase

Introduction

Prostate cancer is the second leading cause of cancer deaths in men. It is not invariably lethal, however, and is a heterogeneous disease ranging from asymptomatic to a rapidly fatal systemic malignancy. In 2005, the American Cancer Society estimates that there will be 234 300 new cases of prostate cancer and that 29 528 men will die of this disease (Jemal *et al*., 2005). Prostate cancers are typically detected through screening based on measurement of the serum prostate specific antigen (PSA) followed by prostate biopsy. The treatment of cancers confined to the prostate gland typically involves radical prostatectomy, external beam radiotherapy or radiotherapy delivered by seed implants (brachytherapy). While many patients with localized disease require no additional treatment, a subgroup will relapse and develop distant metastatic disease. Relapsed patients or patients who present with metastatic disease are treated by withdrawal of androgenic hormones either through medical castration using GNRH agonists or by orchiectomy. While the majority of patients will respond to hormone ablation, responses eventually give way to progressive, hormone-refractory prostate cancer. Additional therapeutic interventions including chemotherapy have some benefit, but of limited duration.

Prostate cancer can be divided epidemiologically into rare hereditary and the vastly more common sporadic forms. Although candidate inherited prostate cancer susceptibility genes have been identified such as the ELAC2, RNASEL, MSR1, NSB1 and CHEK2 genes, the proportion of cases of hereditary prostate cancer attributable to germline mutations in these loci is small and only occasional mutations in these candidate genes have been identified in sporadic prostate cancer (Hsieh *et al*., 2001; Xu *et al*., 2001; Casey *et al*., 2002; Rennert *et al*., 2002; Rokman *et al*., 2002; Xu *et al*., 2002).

The extent of somatic genetic alterations in prostate cancer is not fully understood. Primary prostate tumors are surrounded by stroma and metastatic tumors are typically localized to the bone. These factors contribute to the difficulty in obtaining high-quality tumor-enriched DNA suitable for genetic analysis. Negative mutations studies thus must be interpreted with great caution. Among the best characterized somatic genetic events are amplification of c-MYC and the androgen receptor (AR), mutation of p53, hemizygous deletion at 8p21 thought to target NKX3.1 and loss or mutation of RB1 (reviewed in Sellers and Sawyers, 2002). In 1997, the tumor suppressor gene *PTEN* was cloned from the 10q23 region, a region frequently targeted by loss of heterozygosity (LOH) in advanced cancers (Li and Sun, 1997; Li *et al*., 1997; Steck *et al*., 1997). Inheritance of a mutated germ line allele of PTEN is linked to the development of the related hamartoma syndromes Cowden disease (CD) and Bannayana–Zonana syndrome (BZS) (Liaw *et al*., 1997; Lynch *et al*., 1997; Nelen *et al*., 1997; Marsh *et al*., 1997a). CD is associated with an increased incidence of breast and thyroid malignancies (Marsh *et al*., 1997b); thus, germline mutations or PTEN confer an increased risk of malignancy. As will be detailed further below, somatic inactivation of PTEN is common in a number of cancers including prostate cancer, and over the past 8 years it has become clear that the loss of PTEN and subsequent activation of Akt is a critical event in human prostate cancer, and presents a pathway for rationally targeted molecular therapeutics.

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PTEN – a regulator of the phosphoinositide-3 kinase pathway

PTEN – a PIP3 phosphatase

The *PTEN* gene encodes a dual-specificity phosphatase active against protein substrates (Myers *et al*., 1997). Surprisingly, however, it has much better phosphatase activity against lipid substrates and in particular against the D3 phosphorylated position of phosphoinositide-3,4,5 trisphosphate (PIP3) (Maehama and Dixon, 1998). This lipid is the direct product of the phosphoinositide-3 kinase holoenzyme, suggesting that PTEN might act as a direct antagonist to the PI3K signaling pathway – a known critical oncogenic signaling pathway. Indeed, cells lacking an intact copy of PTEN harbor elevated levels of PIP3.

PI3K is a critical mediator of multiple signaling pathways. Simplistically, receptor tyrosine kinase growth factor receptors become activated and bind to the p85 regulatory subunit. This subunit binds to the catalytic subunit (p110) and activates PI3K. PI3K then phosphorylates the inositol ring of PI4P or PI4,5P2 at the D position to generate PI3,4P2 and PI3,4,5P3, which act as secondary messengers (Cantley and Neel, 1999).

From PIP3 to PI3K and AKT

Important downstream targets of PI3K and of PIP3 include the serine–threonine Akt kinase family (also known as PKB). PIP3, once generated in the plasma membrane, recruits Akt and PDK1 to the plasma membrane through an interaction between the phosphoinositide and the Akt or PDK1 pleckstrin homology domains (PH). Once recruited to the plasma membrane, Akt is phosphorylated and activated by PDK1 (Downward, 1998). Thus, PTEN null cells also harbor constitutively activated levels of Akt. For example, the prostate cancer cell lines PC3 and LNCaP harbor deletions and point mutation of PTEN, rendering each PTEN null. In these cells, basal levels of phosphorylated and hence active Akt exceed the levels of Akt seen in PTEN wild-type cells under conditions of serum stimulation.

Akt promotes both cell growth and cell survival by inactivating its downstream substrates including GSK3, BAD, FOXO and TSC2. Importantly, studies in *Caenorhabditis elegans* and *Drosophilla melanogaster* have linked activation of Akt to regulation of certain FOXO transcription factors and to the activation of mTOR and $p70^{S6K}$. Thus, as one might predict in human cancer cells lacking PTEN substrates of Akt including GSK3, FOXO proteins and TSC2 are also constitutively phosphorylated.

Linking tumor suppression by PTEN to regulation of PI3K signaling

While PTEN has been implicated in regulating non-PI3K pathway functions such as p53, the accumulating evidence supports the notion that transformation resulting from the loss of PTEN is likely mediated through dysregulation of the PI3K pathway. For example, the germline mutant PTEN; G129E retains the ability to dephosphorylate lipid substrates, but selectively lacks the lipid phosphatase activity (Myers *et al*., 1998; Ramaswamy *et al*., 1999). This germline mutation is associated with the identical phenotype as seen with mutations that render PTEN null for both lipid and protein phosphatase activity. In keeping with the central role of PI3K signaling downstream of PTEN inactivation, prostate cancer cells and other cancer cell lines lacking PTEN remain dependent upon activation of the PI3K pathway for growth and survival. Reconstitution of PTEN to such cells either arrests cells in G1 or induces apoptosis. PTEN reconstitution also suppresses the growth of PTEN-null prostate cancer cell lines in soft-agar and in nude mice. These phenotypic reversions also require the PTEN lipid phosphatase activity (Myers *et al*., 1998; Ramaswamy *et al*., 1999). Moreover, antagonizing signaling through the PI3K pathway can also revert the transformed phenotype of PTEN null prostate cancer cells. In a variety of mammalian systems, inactivation of Akt alleles, restoration of Forkhead activity or inhibition of mTOR and $p70^{S6K}$ activities reverses many aspects of the transformed phenotype that results from the loss of PTEN (Nakamura *et al*., 2000; Aoki *et al*., 2001; Neshat *et al*., 2001; Podsypanina *et al*., 2001; Stiles *et al*., 2002). Finally, the requirement for continued PI3K signaling elicited by PTEN loss in cancer cell lines is in keeping with the genetic connections established between PI3K, AKT and PTEN in *D. melanogaster* and *C. elegans*.

Thus, loss of PTEN leads to a continued dependence of PTEN-null cells on PI3K pathway activation. This continued dependence provides a notable therapeutic opportunity.

Somatic mutation of PTEN or PI3K pathway genes in human prostate cancer

Inactivating mutations in PTEN

The discovery of somatic alterations in the PI3K pathway in prostate cancer began with observations of LOH in the region of 10q. This event occurs in CaP with high frequency (30–60%) (Gray *et al*., 1995; Komiya *et al*., 1996) and two distinct commonly LOH regions have been identified at 10q22–q24 and 10q25, respectively, implying the presence of putative tumor suppressor genes at these loci (Komiya *et al*., 1996).

As mentioned above, *PTEN* maps to the 10q23.3 locus and is likely the tumor suppressor gene targeted by this genetic event (Li and Sun, 1997; Li *et al*., 1997; Steck *et al*., 1997). Somatic alterations of *PTEN* are common in other primary tumors including gliomas (Liu *et al*., 1997; Rasheed *et al*., 1997; Wang *et al*., 1997), endometrial cancers (Risinger *et al*., 1997; Tashiro *et al*., 1997), thyroid carcinoma (Dahia *et al*., 1997) and melanoma (Guldberg *et al*., 1997; Tsao *et al*., 1998).

Interest in genetic alterations in PTEN in prostate cancer began with the observation that PC-3 and

LNCaP cell lines (2 of the 3 commonly used prostate cell lines) harbor either a deletion (PC-3) or a point mutation in PTEN (LNCaP) (Li *et al*., 1997; Steck *et al*., 1997). Somatic *PTEN* alterations have been reported for both localized and metastatic prostate cancers. These genetic alterations include homozygous deletions, LOH, and inactivating missense and nonsense mutations. Point mutations in primary tumors were found in one of 40 primary tumors (Dong *et al*., 1998) and in five of 37 primary tumors (Gray *et al*., 1998), while homozygous deletions but not mutations were seen in eight of 60 tumors (Wang *et al*., 1998). Finally, Cairns *et al* reported LOH in 23 of 80 primary tumors with either deletion or mutation of the remaining allele in 10 of the 23 LOH + tumors. Thus, it is reasonable to conclude that a substantial minority ($\sim$15%) of primary tumors harbors PTEN mutations. This is of notable interest, as only a minority of primary tumors are associated with progression to lethal prostate cancer.

Somatic *PTEN* alterations appear more common in metastatic cancers. Suzuki *et al*. (1998) noted that 12 of 19 patients with metastatic disease had a mutation in PTEN in at least one metastatic site. Xenografts derived from metastatic foci have a high rate of PTEN loss and, specifically, homozygous deletion (Vlietstra *et al*., 1998). In keeping with these data, our group has assessed copy number alterations and LOH patterns in primary, hormone-sensitive lymph node metastatic prostate cancer and hormone-refractory metastatic prostate cancer, using high-density (100 K) single-nucleotide polymorphism arrays (Beroukhim R *et al*., unpublished data). As shown in Figure 1, biallelic loss is first seen in lymph node metastases and occurs in 50% of metastatic hormone-refractory prostate cancer. The analysis of larger numbers of primary tumors is required before we can determine whether a fraction of these tumors harbors deletions as well. Together, the bulk of the data suggests that the prevalence of *PTEN* mutation increases in the metastatic disease setting. Similarly, when studied by immunohistochemistry, loss of PTEN protein occurs in approximately 20% of localized prostate tumors. In this setting, PTEN loss is highly correlated with advanced stage and high Gleason grade (McMenamin *et al*., 1999). Thus, there may be a subfraction of primary tumors that lose PTEN and are hence destined to become metatstatic and hormone-independent.

Alterations in Akt in human prostate cancer

While amplification of the AKT1 or AKT2 genes has been noted in pancreatic ductal carcinomas (Cheng *et al*., 1996; Miwa *et al*., 1996; Ruggeri *et al*., 1998) and in ovarian and gastric cancer specimens (Staal, 1987; Cheng *et al*., 1992; Thompson *et al*., 1996), amplification of these loci has not been observed in prostate cancer. To date, activating point mutations have not been described in either AKT1, 2 or 3 in prostate cancer.

The activation of Akt has been studied in prostate cancer specimens using immunohistochemical means to detect phosphorylated Akt (pS473). In some studies, staining was detected in nearly all PIN and invasive prostate cancer samples (Sun *et al*., 2001; Van de Sande *et al*., 2005), while in another study the intensity of pS473 staining was positively correlated, with high preoperative serum levels of PSA (Liao *et al*., 2003) or was significantly greater, with higher Gleason grades 8–10 than PIN (Malik *et al*., 2002; Kreisberg *et al*., 2004). The relationship between IHC detection of Akt activation and PTEN mutation has not been established in prostate cancer.

Alterations in PI3K in human prostate cancer

Amplification (Shayesteh *et al*., 1999) and mutation (Samuels *et al*., 2004) of the gene encoding the catalytic subunit of the type 1 PI3K alpha subunit (PIK3CA) have been described as frequent somatic events in ovarian cancer, in breast cancer, hepatocellular carcinoma and glioblastoma among many cancer types. (Bachman *et al*., 2004; Broderick *et al*., 2004; Campbell *et al*., 2004). In prostate cancer, however, neither amplification nor mutation has been reported to date.

Alterations in IGFI in human prostate cancer

An association between plasma levels of IGF-1 and the risk of death from prostate cancer has been observed in prospective, population-based cohort studies (Chan *et al*., 1998; Wolk *et al*., 1998). Here, those men who are in the top quintile of IGF1 levels have a statistically

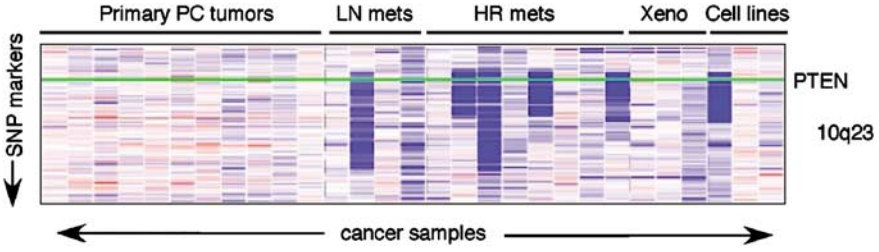

Figure 1 High-density single-nucleotide polymorphism (SNP) array analysis reveals homozygous deletions of the PTEN gene in prostate cancer samples. Each column represent one tumor or cell line sample (as indicated), while each row represents a single SNP marker. Only markers shown in the 10q23 interval are depicted. Increasing shades of blue represents gene copy-loss while increasing shades of red represent gene copy-gain. The genomic position of the PTEN gene is indicated by the green line. Abbreviations: PC – prostate cancer, LN – lymph node, xeno – xenografts, HR – hormone refractory. Genomic DNA from each sample was isolated, and processed for SNP array hybridization as previously described (Garraway *et al*., 2005)

significant increase in the risk of death from prostate cancer. More recent studies obtained in the so-called post-PSA era of prostate cancer diagnosis have failed to find these same associations. Thus, there remains a lack of clarity surrounding this finding.

Murine models of prostate cancer based on PI3K pathway activation

PTEN knockout mice

Conventional deletion of both alleles of *Pten* leads to developmental defects and death at embryonic days 6.5–9.5 days (Di Cristofano *et al*., 1998; Podsypanina *et al*., 1999). *Pten* heterozygous (+/−) mice develop prostatic intraepithelial neoplasia with nearly 100% penetrance, but these lesions apparently do not progress to macroinvasive cancers (Di Cristofano *et al*., 1998; Podsypanina *et al*., 1999). The viability of the *Pten*+/− mice is compromised as a result of lymphoproliferation and tumors of intestines, mammary, thyroid, endometrial and adrenal glands. Thus, it has been difficult to look at the resulting prostate phenotype in older aged mice.

Heterozygosity of *Pten* also cooperates with a number of engineered secondary events to enhance the phenotype. Heterozygous or homozygous loss of *Cdkn2b* ($p27^{Kip1}$), *Nkx3.1* and *Ink4a*/$p19^{arf}$ all exacerbate the PTEN prostatic phenotype. For example, *Pten*+/− mice, in the background of the *Cdkn2b*−/− genotype, develop prostate cancer within 3 months with 100% penetrance (Di Cristofano *et al*., 2001). PIN was observed in *Pten*+/−, *Ink4a*/$p19^{arf}$+/− or −/− earlier than in *Pten*+/− mice alone; however, progression to invasive cancer was not observed (You *et al*., 2002). PIN was observed earlier in *Pten*+/−,*Nkx3.1*+/− than in *Pten*+/− mice alone and invasive cancers with lymph node metastases are found in *Pten*+/−,*Nkx3.1*+/−, *Cdkn2b*+/− mice (Abate-Shen *et al*., 2003; Gao *et al*., 2004). Finally, despite the convergence of PTEN and TSC2 on a common downstream signaling pathway (mTOR) reduction of *Tsc2* cooperates to induce invasive prostate cancers in *Pten*+/− mice (Ma *et al*., 2005a).

LoxP-PTEN knockout mice

To determine the consequence of prostate-specific deletion of *Pten*, mice harboring floxed alleles of *Pten* (Lesche *et al*., 2002) have been generated and intercrossed with mice bearing a transgene directing the constitutive prostate-specific expression of Cre-recombinase (*ARR2PB*-Cre). In mice lacking both alleles of *Pten,* PIN develops with earlier onset than in *Pten*+/− mice and leads to invasive prostate cancer and ultimately to metastatic cancer (Trotman *et al*., 2003; Wang *et al*., 2003). Similarly, homozygous deletion of *Pten* achieved using a PSA promoter-driven Cre-recombinase leads to invasive prostate cancer with a 100% penetrance (Ma *et al*., 2005b) and similar pictures of progression were seen in mice bearing *MMTV*-Cre and *Pten* flox alleles (Backman *et al*., 2004).

These data strongly support the role of PTEN as a tumor suppressor, with particular relevance to prostate cancer initiation and progression.

Prostate-specific Akt transgenic mice

A transgenic line expressing a myristoylated and hence constitutively activated form of human Akt-1 was generated, in which the rat probasin promoter was used to restrict expression to the prostate (Majumder *et al*., 2003). In this model, activated AKT1 is spatially overexpressed in the ventral and lateral prostates, starting as early as postnatal day 2. The overexpression and activation of downstream molecules results in the development of dysplastic lesions with severe atypia, histopathological features consistent with PIN. AKT activation also led to changes in gene expression that are also known to occur in human prostate cancers. Notable among the upregulated transcripts was prostate stem cell antigen (PSCA), a gene that is expressed in prostate ductal tips during prostate development (Reiter *et al*., 1998). In human prostate cancers, PSCA is expressed in almost all cases of high-grade PIN and is overexpressed in approximately 40% of local and as many as 100% of bone metastatic prostate cancers (Gu *et al*., 2000).

The PIN phenotype does not progress to cancer, but 30–40% of older Akt-transgenic mice develop a protuberant abdomen as a result of a bladder outlet obstruction. In contrast to the results obtained with loss of function alleles of *Pten*, Tg-Akt1 did not develop invasive or metastatic prostate cancer. This important difference in phenotype may reflect the biologic differences between activating only Akt1 as opposed to inactivating PTEN, with the subsequent activation of PI3K and Akt. Alternatively, strain differences and hence germline genetic modifiers could, at least in part, account for some of these differences in the Akt1 transgenic mice maintained in the FVB background.

Genetic suppression of the PTEN phenotype in murine systems

The development of therapeutics for reversing treating of PTEN null tumors has been an area of intensive investigation. This results primarily from the array of drugable kinases that are suitable targets downstream of PTEN loss. To date, limited experiments have been carried out in mice to try and ascertain whether genetic deletion of any given pathway component (e.g. Akt or PI3K) is sufficient to suppress the PTEN phenotype. First, studies of teratoma formation suggest that Akt-1 is a major effector of the proliferation and tumor phenotype in *PTEN* homozygous (−/−) ES cells (Stiles *et al*., 2002). It has been more difficult to address the necessity of PI3K downstream of PTEN loss as mice lacking catalytic subunits of p110 are not viable; however, it has been possible to examine the requirement for the regulatory subunits of PI3K. Specifically, loss of the p85α and p85β subunits of PI3K in *Pten*+/−

mice does not alter the PIN formation, but a fraction of the proliferating cells in PIN is reduced in *Pten*+/− *p85β*−/− mice (Luo *et al*., 2005a).

Akt, a regulator of mTOR and its role in prostate cancer

The mTOR pathway is an important component of the downstream signaling cascade that is dysregulated by loss of function mutations in PTEN. These connections between activated Akt and activation of mTOR, likely involve Akt-dependent inactivation of TSC2 or proceed through the Akt or PDK1-dependent activation of $p70^{S6K}$. In either case, cells lacking PTEN or harboring activated alleles of Akt have high levels of mTOR activity and a resulting dysregulation of cell size, organ size and cell growth controls.

The mammalian target of rapamycin (mTOR) was identified after the discovery of its yeast homologs TOR1 and TOR2 (Brown *et al*., 1994; Chiu *et al*., 1994; Sabatini *et al*., 1994). mTOR is a member of the atypical protein kinase family and phosphorylates substrates critical for protein synthesis, including ribosomal subunit S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (Schmelzle and Hall, 2000). Thus, the output of mTOR signaling is likely mediated through regulation of protein translation. How mTOR activation might lead to cellular transformation is still not completely clear. Downstream of mTOR broad alterations in protein translation might account for oncogenesis. Alternatively, specific critical proteins that are regulated through translation might be deregulated and may contribute to the required oncogenic signals. For example, it has also been proposed that dysregulation of the protein synthesis machinery through mTOR may generate cell-cycle progression signals that contribute to cancer (Ruggero and Pandolfi, 2003). Another potential target of mTOR is PP2A, which is downregulated by small T-antigen and is important for the transformation of human cells with SV40 T-antigens and Ras (Hahn *et al*., 2002). mTOR phosphorylates PP2A *in vitro* likely leading to downregulation of PP2A preventing the dephosphorylation of 4E-BP1 (Peterson *et al*., 1999). Thus, it is possible that PP2A is a critical target of mTOR-mediated signals.

In this vein, emerging data suggest that Hif1α stabilization may play an important role downstream in the induction of the neoplastic phenotype *in vivo*, and that mTOR inhibition, at least in part, reverts aspects of transformation through regulation of Hif1α levels (Hudson *et al*., 2002; Brugarolas *et al*., 2004; Majumder *et al*., 2004). Hif1α transcriptional targets are constitutively activated in the Akt-dependent prostatic intraepithelial neoplasia model, and this deregulation is completely mTOR dependent. Among the targets of Hif1α transcription are included nearly all the members of the glycolysis pathway from hexokinase to lactate dehydrogenase. An important implication is that the regulation of glycolysis and glucose uptake may likewise play an important role in the growth of such tumors.

With respect to Hif1α regulation, both Akt-dependent/mTOR-independent pathway and Akt dependent/mTOR-dependent pathways have been described (reviewed in Semenza, 2003; Abraham, 2004). Recent data suggest that hypoxia-induced activation of Hif1α requires mTOR activity (Zhong *et al*., 2000; Hudson *et al*., 2002), that insulin activates Hif1α through the Akt/mTOR-dependent pathway (Treins *et al*., 2002) and that in the setting of loss of TSC2, Hif1α protein and mRNA levels are elevated, leading to upregulated expression of Hif1α target genes (Brugarolas *et al*., 2003). Elevated Hif1α activity is, in this setting, reversed by mTOR inhibition (Brugarolas *et al*., 2003), but the mechanism leading to mTOR-dependent elevated Hif1α activity remains unclear.

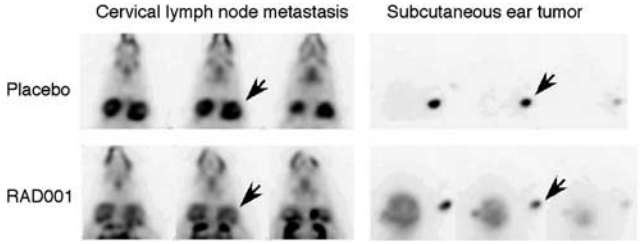

Figure 2 ^{18}FDG-PET uptake is blocked by two doses of the rapamycin derivative RAD001 in primary and metastatic tumors. Murine cancer cells were subcutaneously implanted in the mouse ear and allowed to grow in the implantation sites and metastasize to cervical lymph nodes. Tumor bearing mice were treated with either placebo or RAD001 at a dose of 10 mg/kg body weight for 2 days and glucose uptake was determined by ^{18}FDG-PET analysis. Arrows indicate the position of the cervical lymph node metastasis (left panel) and subcutaneous ear tumor (right panel). This figure was generously provided by Paul McSheehy, Novartis Institute of Biomedical Research, Oncology, CH-4002, Basel, Switzerland

Glucose uptake and in particular hexokinase activity can be 'sensed' or imaged *in vivo* using the radiotracer ^{18}Fluorodeoxyglucose and positron emission tomography (^{18}FDG-PET). Generally, ^{18}FDG-PET uptake by tumors is thought to simply reflect upregulated metabolic activity; however, the regulation by mTOR suggests that genetic alteration of the pathway may specifically turn on glycolytic enzymes. Indeed, preclinical proof-of-concept experiments have shown that ^{18}FDG-PET uptake can be blocked by two doses of the rapamycin derivative RAD001 (Figure 2) (McSheehy *et al*., 2005). This provides the opportunity to use ^{18}FDG-PET as an *in vivo* pharmacodynamic marker for mTOR inhibitors.

Strategies for blocking the PI3K/Akt/mTOR pathway in prostate cancer

Introduction

The frequent occurrence of inactivating mutations in the PTEN tumor suppressor in hormone-refractory prostate cancer has provided one of the few genetically defined in-roads to cancer therapeutics in prostate cancer. The PI3K pathway presents a number of attractive kinase targets for small molecule development that will be discussed below. Furthest along in clinical development

are the inhibitors of mTOR, all of which are derivatives of rapamycin.

IGF1R inhibition

IGF1R has long been known as a critical survival and proliferation signal transducer. While genetic alterations in cancer have not yet been observed, murine fibroblasts that lack IGFIR are resistant to the transforming activities of a number of oncogenes including SV40 large-T antigen (Coppola *et al*., 1994). These latter data suggest that IGFIR is required for transformation and thus might be a suitable drug target. The IGFI/IGFIR axis is clearly important in human prostate development and in the development of prostate cancer. Indeed, constitutive secretion of IGFI itself in transgenic animals is sufficient to induce prostate cancer (DiGiovanni *et al*., 2000). Examination of IGFI−/− mice has also revealed that IGFI is required for the normal development of the murine prostate (Ruan *et al*., 1999).

IGFIR small-molecule inhibitors are in development and have reported activity against myeloma, small cell lung cancer and certain sarcomas; however, activity against prostate cancer has not been described to date (Garcia-Echeverria *et al*., 2004; Mitsiades *et al*., 2004; Scotlandi *et al*., 2005; Warshamana-Greene *et al*., 2005). It is also not clear whether such inhibitors would have preferential activity against PTEN-null cells or conversely whether loss of PTEN might render cells resistant to upstream IGFIR inhibition. Similarly, humanized selective antibodies directed against IGFIR are also in development, but whether such antibodies will have therapeutic efficacy remains to be seen (Burtrum *et al*., 2003; Maloney *et al*., 2003; Cohen *et al*., 2005).

PI3K inhibition

There are ongoing efforts to develop kinase inhibitors against the catalytic subunits of PI3K. Early leads in this area include the commonly used laboratory tool compounds wortmannin and LY294002, which target the p110 catalytic unit of PI3K. These molecules have relatively broad specificity and short *in vivo* half-life and are poorly suited for clinical development. Second generation PI3K inhibitors, though not widely published appear to have much improved isoform specificity and also improved pharmacologic properties and activity in xenograft models (Ward *et al*., 2003; Workman, 2004). Again, however, whether there will be enhanced sensitivity in cells lacking PTEN or whether there is activity in prostate cancer models remains to be seen. These agents have not yet reached the clinic.

An alternative strategy for interrupting PI3K signaling is to block recruitment of the PI3K holoenzyme to receptor tyrosine kinases by disrupting the phosphotyrosine binding of the SH2-domain of the p85 subunit of PI3K. Here, peptidomimetics have been successfully used to block this association (Eaton *et al*., 1998), but will likely require significant optimization prior to *in vivo* administration.

AKT inhibition

Inhibition of Akt remains an attractive therapeutic approach to interdiction of the PI3K pathway. Despite serious efforts directed at building ATP-competitive kinase inhibitors, no selective small molecules have made it to the clinic. High potency inhibitors have been described (Thimmaiah *et al*., 2005; Luo *et al*., 2005b) and appear to have antitumor activity in xenografts models, although with some evidence of metabolic toxicities. In addition, a set of allosteric inhibitors that requires the PH domain of Akt for activity have been described (Barnett *et al*., 2005; Lindsley *et al*., 2005) and *in vitro*, induce apoptosis in the PTEN-null LNCaP prostate cell line. Finally, a number of 'akt' or pathway inhibitors have been described including calmodulin inhibitors (Kau *et al*., 2003), myo-inositol derivatives (Meuillet *et al*., 2004; Tabellini *et al*., 2004) and phosphatidylinositol ether analogs (Gills and Dennis, 2004) that inhibit the activation of Akt as measured by inhibition of Akt phosphorylation itself (as opposed to direct activation of Akt kinase activity). These latter agents might act either on Akt recruitment or on known or novel upstream activators of Akt. To date, there is no significant clinical experience with these agents in prostate cancer patients.

HSP90 inhibition

It appears that either events that lead to the activation of protein kinases, or kinases bearing activating mutations become particularly dependent on chaperone function for appropriate folding and activity. Notable in this regard is that phosphorylated and hence activated

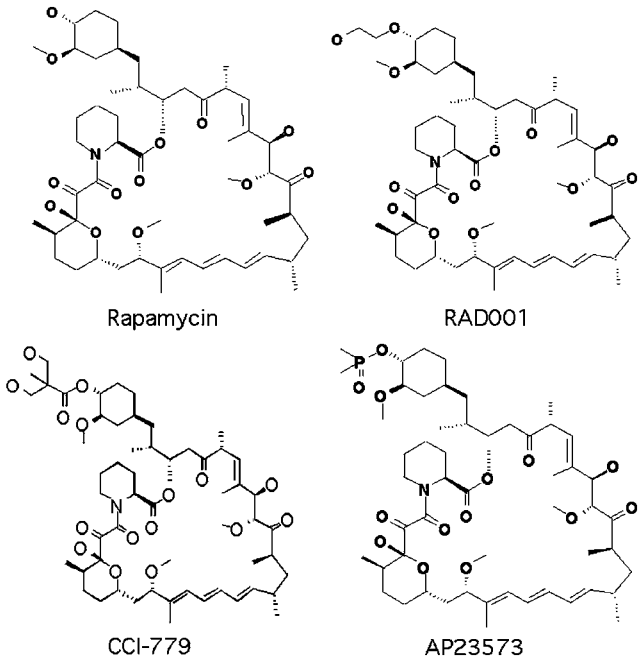

Figure 3 Structures of rapamycin and the rapamycin-derivatives in clinical development

Table 1 Current clinical status of PI3K/Akt/mTOR pathway inhibitors in cancer

Drug	*Manufacturer*	*Disease*	*Trial status*
CCI-779	Wyeth-Ayerst Laboratories	Malignant gliomas	Phase I/II
		Metastatic breast cancer	Phase III
		Androgen-independent prostate cancer	Phase II
RAD001	Novartis Pharmaceutical	Advance non-small-cell lung cancer	Phase I/II
		Endometrial cancer	Phase II
		Recurrent glioblastoma	Phase I/II
AP 23573	Ariad Pharmaceuticals	Progressive and recurrent gliomas	Phase I

Akt likewise appears to require the function of the HSP90 chaperone for continued activity (Sato *et al*., 2000; Hostein *et al*., 2001; Basso *et al*., 2002; Solit *et al*., 2003; Gills and Dennis, 2004). Geldanamycin or its derivative 17-AAG are ansamycins that selectively inhibit HSP90 function by occupying the nucleotide binding site. The ansamycins appear to have anticancer activity and have prompted the development of new series of HSP90 inhibitors with improved pharmacologic properties and facile syntheses (reviewed in Chiosis *et al*., 2003; Cheung *et al*., 2005).

Recently, phase I data were reported for 17-AAG. Here, the dose-limiting toxicities were diarrhea and hepatoxicity. There were two patients with melanoma who had evidence for disease stabilization and no patients with prostate cancer were treated in this study (Banerji *et al*., 2005).

mTOR inhibition

Rapamycin, first isolated from *Streptomyces hygroscopicus,* binds to FKBP12 (also known as FK506-binding protein) and induces binding to and inactivation of mTOR. Cell lines harboring inactivating mutations in PTEN are particularly sensitive to rapamycin or the derivative CCI-779 (Neshat *et al*., 2001). Similarly, chicken fibroblasts transformed by activating alleles of AKT or PI3K also have increased sensitivity to these agents (Aoki *et al*., 2001). *In vivo*, treating *Pten* + /− null mice with CCI-779 reduces the number of intestinal lesions (Podsypanina *et al*., 2001) and the treatment of Akt transgenic animals with RAD001 completely reverts the PIN phenotype to normal (Majumder *et al*., 2004). These experiments have encouraged the clinical development of mTOR inhibitors in the context of PTEN or PI3K pathway alterations. However, the mTOR-dependent regulation of Hif1α also raises the possibility that rapamycin or its derivatives might have a role as anti-angiogenic agents.

Current status of mTOR inhibitors in prostate cancer

Three derivatives of rapamycin CCI-779, RAD001 and AP 23573 (see Figure 3) either have completed or are completing phase I trials (Table 1). A phase II trial of CCI-779 was initiated in prostate cancer using intravenous weekly dosing. Toxicity may have resulted in the premature closure of this trial and no data have been reported in the published literature. A number of new phase II trials are underway, including a combination trial of Gefitinib and RAD001 in prostate cancer, as well as a Taxotere and RAD001 trial. Both of these trials will use ^{18}FDG-PET imaging as a pharmacodynamic end point. It will likely be some time before we know whether mTOR inhibition will have therapeutic value in selected patients or in combination with second agents.

Summary

The genetic evidence strongly supports the role of PTEN mutation and hence AKT activation in metastatic or high-grade prostate cancers. Therapeutics for these aggressive forms of prostate cancer are severely lacking and agents targeting this pathway are likely to find a role in the management of prostate cancer. The major hurdles remain the discovery, optimization and clinical development of small molecule inhibitors for the major kinases dysregulated by PTEN loss, namely AKT and PI3K.

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